

THE ROLE OF THYMIC STROMAL LYMPHOPOIETIN IN PULMONARY FIBROSIS

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Declaration

I confirm that the work contained in this thesis is entirely my own.

To Nichuviü, Istara and Roshana

ABSTRACT

Thymic stromal lymphopoietin (TSLP) is an IL-7 like cytokine that has recently emerged as being critical in promoting T-2 mediated inflammatory responses in atopic disease via its unique activation of dendritic cells (DCs). However, its role in idiopathic pulmonary fibrosis (IPF), a non-allergen driven disease characterised by excessive fibroblast activation and a pro-fibrotic T-2 immune phenotype, is unknown.

TSLP has traditionally been regarded as an epithelial-derived cytokine targeting cells of a haematopoietic lineage. However, the present work identifies fibroblasts as a potentially important source and cellular target in IPF. Primary human lung fibroblasts (pHLFs) express and release TSLP in a JNK/c-Jun dependent manner following exposure to TNF- α , a master cytokine strongly implicated in the pathogenesis of lung fibrosis. Moreover, this thesis demonstrates for the first time that lung fibroblasts express a functional TSLP-TSLPR signalling axis; following exposure to TSLP, these cells release the pro-fibrotic chemokine, CCL2, in a STAT3-dependent manner, thereby promoting chemotaxis of human monocytes. The work presented here further identifies TSLP as a key mediator of local DC activation and the generation of a T-2 immune phenotype in the bleomycin model of lung injury and fibrosis. However, neutralisation of TSLP activity *in vivo* did not attenuate bleomycin-induced fibrosis, suggesting that despite its essential role in DC activation and T-2 polarisation, TSLP may not play a significant role in the subsequent development of fibrosis in this model. Taken together, these studies extend our current understanding of TSLP as a master regulator of T-2 immune responses beyond that of allergic inflammatory conditions. The human expression and functional studies performed during the course of this PhD do not rule out a potential pathogenic role for TSLP in IPF. Extended studies are warranted to explore these observations further.

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LIST OF ABBREVIATIONS

ACM	alveolar -capillary membrane
AEC	alveolar epithelial cell
AP	activator protein
BALF	broncho-alveolar lavage fluid
BLM	bleomycin
BM	basement membrane
BSA	bovine serum albumin
CCL	chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	cluster of differentiation
COX	cyclo-oxygenase
CTGF	connective tissue growth factor
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DD	death-domain
DMEM	dulbecco's modified eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
eATP	extracellular adenosine triphosphate
EBV	epstein barr virus
ECM	extra cellular matrix
EDTA	ethylenediaminetetracetic acid
ELISA	enzyme-linked immunoassay
EMT	epithelial-mesenchymal transition
ERK	extracellular signal-regulated kinase
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
HASMC	human airway smooth muscle cells
HGF	hepatocyte growth factor
HLF	human lung fibroblast
HPLC	high pressure liquid chromatography
HRCT	high resolution computed tomography

ICAM	intercellular adhesion molecule
IFN	interferon
IIP	idiopathic interstitial pneumonia
IKK	I κ B kinase
ILD	interstitial lung disease
IPF	idiopathic pulmonary fibrosis
JAK	janus kinase
JNK	c-Jun amino-terminal kinase
kDa	kilodalton
KGF	keratinocyte growth factor
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MEK	mitogen activated protein kinase kinase
MHC	major histocompatibility complex
MLF	murine lung fibroblast
NHBE	normal human bronchial epithelial cell
NK	natural killer
NSIP	non-specific interstitial pneumonia
OVA	ovalbumin
PBS	phosphate buffered saline
PF	pulmonary fibrosis
PFA	paraformaldehyde
PGE ₂	prostaglandin E ₂
PMA	phorbol myristate acetate
qRT-PCR	real time quantitative reverse transcription polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of mean
SIGN	specific intercellular adhesion molecule-3-grabbing non-integrin
SMA	smooth muscle actin
SODD	silencer of death domain
STAT	signal transducer and activator of transcription
TACE	TNF- α converting enzyme
TCR	T-cell receptor
TFG- β	transforming growth factor- β

Th	T-helper
TRADD	tumour necrosis factor receptor type 1-associated death domain protein
TRAF	TNF receptor-associated factor
TSLP	thymic stromal lymphopoietin
TSLPR	thymic stromal lymphopoietin receptor
UIP	usual interstitial pneumonia

CHAPTER 1: INTRODUCTION

1.1. Pulmonary Fibrosis

Pulmonary fibrosis (PF) represents the end stage of a heterogeneous group of conditions that are characterised by the excessive deposition of extracellular matrix (ECM) proteins within the pulmonary interstitium. This interstitium is composed of the alveolar walls, including epithelial and endothelial cells, their septae and associated peri-lymphatic and peri-bronchiolar connective tissue. It is across this critical space that gas exchange occurs. The deposited ECM proteins, such as collagen, ultimately replace normal functional tissue in a disorganised fashion. This fibrosis is generally progressive and, in many cases, leads inexorably to respiratory failure, due to obliteration of functional alveolar units, and premature death.

PF, to a greater or lesser degree, is a hallmark of the interstitial lung diseases (ILDs). Although individual disease patterns within this group of conditions share a number of clinical, radiographic and pathological features, mechanisms of disease and prognoses are often very different. A small number of ILDs have well defined aetiologies relating to occupational factors (eg. asbestosis) or drug exposure (eg. methotrexate); some are associated with systemic disorders such as rheumatoid arthritis or systemic sclerosis. The ILDs with known aetiologies have been grouped in **Table 1.1**. However, the vast majority are idiopathic, and are termed the Idiopathic Interstitial Pneumonias (IIPs).

Ever since the fibrotic sequelae of ILDs were first described towards the end of the 19th century, classification of IIPs has been marred by controversy and difficulty (Maher *et al.*, 2007). However in 2002, the American Thoracic Society (ATS) and European Respiratory Society (ERS) consensus panels reclassified IIPs into seven distinct disease entities, distinguishable by their clinical, radiological and histological patterns, as illustrated in **Table 1.2**.

1.2. Idiopathic Pulmonary Fibrosis

Idiopathic pulmonary fibrosis (IPF) accounts for almost half of all the IIPs (ATS, 2000). In the UK, it has a reported incidence of 4.6 per 100000 people in the general population (Gribbin *et al.*, 2006), a figure which has increased annually by 11% between 1991 and 2003 (Gribbin *et al.*, 2006). In the UK alone, around 5000 new cases are currently diagnosed each year and IPF accounts for over 1500

Aetiology		Clinical example
Systemic disease	Collagen vascular disease	Systemic sclerosis
		Rheumatoid arthritis
		Sjorgren's disease
		Poly/dermatomyositis
Exposures	Immunodeficiency	Common variable immunodeficiency
	Environmental	Hypersensitivity pneumonitis
		Asbestosis
		Berylliosis
	Occupational	Silicosis
		Amiodarone
Genetic	Drug induced	Methotrexate
		Familial interstitial pneumonia
		Hermanski-Pudlack syndrome

Table 1.1. Clinical classification of ILDs with known aetiologies (adapted from Laurent and Shapiro, 2007)

deaths per year (Hubbard *et al.*, 1996), representing a disease burden comparable to small cell lung cancer. It is more common in men, and its incidence increases with age. There is no geographical pattern of disease distribution or racial predisposition. A number of clinical, radiological and physiological parameters have been identified as predictors of worse outcome – these include progressive dyspnoea, oxygen desaturation during a 6 minute walk test, the presence and extent of honeycombing on high resolution computed tomography (HRCT) of the thorax and the presence of pulmonary hypertension.

The classical histological pattern of IPF is one of usual interstitial pneumonia (UIP): areas of normal lung are interspersed with regions of dense fibrosis, honeycombing and fibrotic foci (**Figure 1.1.**). Honeycombing occurs as a result of the destruction of lung parenchyma leading to enlarged and distorted airspaces. Fibrotic foci are aggregates of highly synthetic myofibroblasts often observed within the mura of microscopic honeycomb lesions underlying injured and reparative epithelium, as well as within the interstitium – these foci represent accumulations of fibroblasts and myofibroblasts within organizing ECM, and their presence and distribution, together with the spatial and temporal heterogeneity of epithelial damage and ECM deposition is crucial to defining a UIP pattern (Katzenstein *et al.*, 1998). This is in contrast, for example, to the histological pattern of non-specific interstitial pneumonia (NSIP) in which honeycombing is absent and a marked inflammatory interstitial infiltrate is observed. The spatial heterogeneity of UIP is similarly seen on high-resolution CT scans of IPF lung, and the patchy, subpleural, predominantly lower lobe reticular opacities and honeycombing correlates well with UIP, often obviating recourse to a lung biopsy to achieve a definitive diagnosis.

Following diagnosis, although the clinical course of IPF can be extremely variable, the median survival is less than 3 years (Kim *et al.*, 2006a) – this is the poorest prognosis of all the IIPs, and reflects the progressive replacement of functional alveolar units with dense fibrotic tissue. The disease appears to be resistant to conventional anti-inflammatory and immunosuppressive treatment, but despite this, current gold standard therapy comprises low dose steroids and a steroid sparing immunosuppressive agent such as azathioprine, together with the recent addition of N-acetylcysteine (Demedts *et al.*, 2005). Interestingly, a recent study demonstrated that pirfenidone, a novel anti-inflammatory and anti-fibrotic drug, reduces the

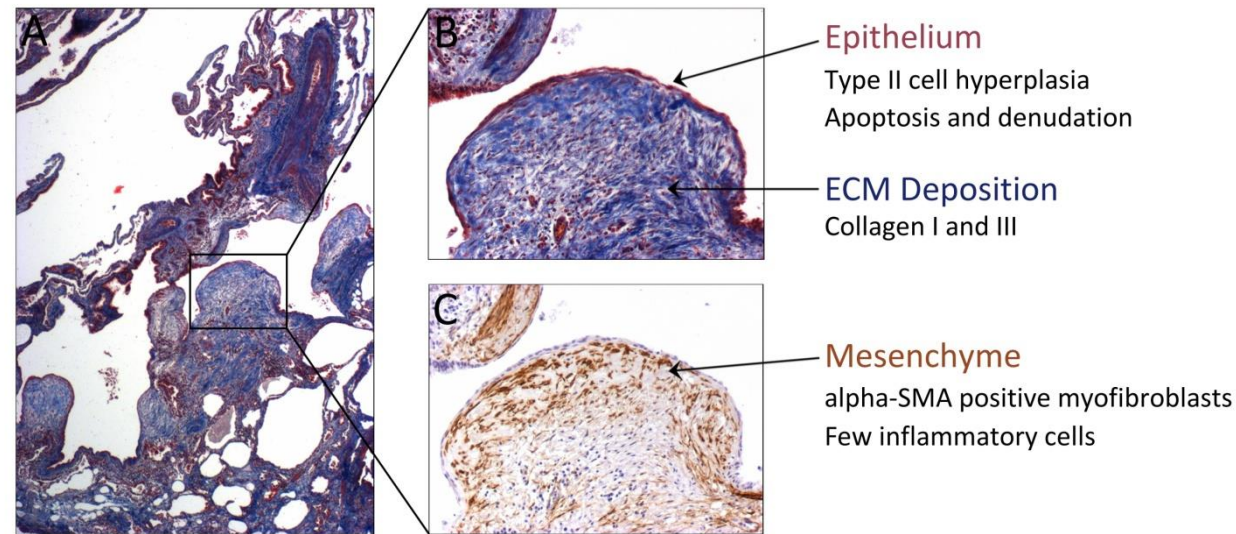


Figure 1.1.

Fibrotic foci – a histological hallmark of idiopathic pulmonary fibrosis. (A) Histological analysis of human IPF tissue reveals the presence of dense collagen deposition within the interstitium (Martius Scarlet Blue staining; original magnification $\times 10$). Fibroblastic foci are revealed as accumulations of fibroblasts and α -SMA⁺ myofibroblasts, which are highly synthetic for collagen and have a contractile phenotype (B: Martius Scarlet Blue staining; C: immunohistochemistry for α -SMA. Original magnification $\times 20$). The overlying epithelium is often hyperplastic, with frequent apoptosis and areas of denudation. The presence and distribution of fibrotic foci, together with the spatial and temporal heterogeneity of the pathology is crucial to defining a UIP pattern. [reproduced from (Datta *et al.*, 2011)]

Histological pattern	Diagnosis based on clinical, radiological and histological findings
Usual interstitial pneumonia (UIP)	Idiopathic pulmonary fibrosis (IPF)
Non-specific interstitial pneumonia (NSIP)	Non-specific interstitial pneumonia (NSIP) ¹
Organizing pneumonia	Cryptogenic organizing pneumonia
Diffuse alveolar damage	Acute interstitial pneumonia
Respiratory bronchiolitis	Respiratory bronchiolitis-associated interstitial lung disease
Desquamative interstitial pneumonia	Desquamative interstitial pneumonia
Lymphoid interstitial pneumonia	Lymphoid interstitial pneumonia

Table 1.2. Classification of Idiopathic Interstitial Pneumonias.

(Table adapted from: American Thoracic Society / European Respiratory Society International multidisciplinary consensus classification of IIPs, 2002)

¹ NSIP can be further sub-divided into cellular and fibrotic NSIP (Nicholson et al., 2000)

deterioration in lung function in IPF patients (Noble *et al.*, 2011). The precise molecular mechanism of action of pirfenidone remains unknown, though its anti-fibrotic potential may relate to its ability to inhibit fibroblast proliferation and extracellular matrix deposition (Di Sario *et al.*, 2002; Hewitson *et al.*, 2001), which may be secondary to inhibition of pro-fibrotic cytokine expression (Gurujeyalakshmi *et al.*, 1999; Iyer *et al.*, 1999).

The aetiology of IPF remains unknown, although a number of risk factors have been identified. For example, cigarette smoking has been associated with an increased risk of developing IPF (Baumgartner *et al.*, 1997), as have certain latent viral infections, including EBV and herpesvirus (Kelly *et al.*, 2002; Tang *et al.*, 2003). Three percent of IPF patients appear to have a familial form, and gene polymorphisms of tumour necrosis factor (TNF)- α and transforming growth factor beta (TGF)- β 1, as well as mutations in surfactant protein C, appear to confer an increased risk of developing IPF (Lawson *et al.*, 2004; Whyte *et al.*, 2000; Xaubet *et al.*, 2003). However, as only a small number of those individuals exposed to known risk factors develop IPF, the true aetiology is likely to be multi-factorial.

1.3. Pathogenesis of pulmonary fibrosis

Although significant advances have been made in our understanding of the pathogenesis of pulmonary fibrosis, the specific molecular and cellular mechanisms that contribute to disease progression remain unclear. Much of our understanding is derived from animal models of pulmonary fibrosis, the commonest of which involves the administration of bleomycin (BLM) to rodents to initiate lung injury, inflammation and subsequent fibrosis. This model was employed during the course of this PhD to study the pathogenesis of lung fibrosis *in vivo*, and will now be discussed in more detail.

1.3.1. The bleomycin model of lung injury and fibrosis

Lung fibrosis is observed in mice challenged with bleomycin via the intravenous (i.v.), intra-tracheal (i.t.), intra-peritoneal (i.p.) or oropharyngeal (o.p.) routes (Chua *et al.*, 2005). Bleomycin binds to dsDNA and initiates DNA scission in an oxygen-dependent manner *in vitro*; these findings are mirrored in animal models of bleomycin-induced lung injury, in which bleomycin challenge results in persistent and extensive DNA damage to lung cells (Harrison *et al.*, 1989) resulting in epithelial injury, reflected by epithelial cell apoptosis. While the i.v. route may reflect the nature of lung fibrosis observed as a side-effect in humans receiving bleomycin

as part of chemotherapy, the development of fibrosis in mice occurs over a relatively long time-frame, rendering it unsuitable to work within the confines of the current studies. A similar issue is faced when employing an i.p. approach, which is additionally associated with increased morbidity. Bleomycin challenge to mice, via the airways, results in an initial non-allergen driven epithelial injury, with the development of a neutrophilic and lymphocytic inflammatory infiltrate during the first week (Janick-Buckner *et al.*, 1989a). The transition to fibrosis is usually observed after two weeks (Scotton *et al.*, 2009). Histological characteristics of BLM-induced fibrosis in rodents include epithelial cell injury with reactive hyperplasia, basement membrane damage and interstitial, as well as intra-alveolar, fibrosis (Usuki *et al.*, 1995). Furthermore, discrete clusters of mesenchymal cells are often observed in areas of repairing lung, reminiscent of fibrotic foci present in IPF. I.t. challenge is perhaps the most widely used, but does result in a bronchocentric distribution of fibrosis, in contrast to the peripheral and subpleural pattern of matrix deposition observed in IPF (Scotton *et al.*, 2010). This pattern is mirrored more faithfully following o.p. challenge in the host laboratory. Fibrotic lung lesions in rodents have been reported to have completely resolved by 6 weeks following a single intra-tracheal (i.t.) bleomycin challenge (Chung *et al.*, 2003; Degryse *et al.*, 2010). In contrast data from micro-CT imaging of murine lung *ex-vivo* suggest that fibrosis is maintained at 6 months following o.p. bleomycin-induced injury (unpublished data, Dr. C Scotton), more in keeping with the non-resolving nature of IPF. This lack of concordance may reflect subtle permutations of the model dependent on route of BLM administration (Chua *et al.*, 2005) , but highlights a significant shortcoming in translating results obtained from this model to clinical outcomes in IPF (Scotton *et al.*, 2010). Regardless of the issue of resolution, the lack of disease progression following single dose BLM injury represents a crucial difference between BLM-induced fibrosis in mice and IPF and, together with a lack of restrictive physiology following injury in mice, highlights deficiencies of the model. Nonetheless, this model has proved invaluable in allowing the identification of a number of key pathways and cytokines involved in the fibroproliferative response to lung injury *in vivo*, and as such was employed in the current studies outlined in **Section 1.9**.

1.3.2. *Current hypotheses regarding the pathogenesis of idiopathic pulmonary fibrosis*

An early and consistent feature of pulmonary fibrosis in humans is a change in the phenotype of the alveolar epithelial cell (AEC), suggesting that alveolar epithelial cell injury is a critical early step in the pathogenesis of pulmonary fibrosis (Chilosi *et al.*, 2002; Kasper *et al.*, 1996). The exact nature of this injury is unclear, but as alluded to earlier, the fibroproliferative response to injury is likely to be due to a combination of host-specific, genetic and environmental factors.

Fibroblastic foci are the characteristic histological feature of UIP/IPF, and generally underlie areas of damaged or altered epithelium. They are composed of aggregates of myofibroblasts, which are the key effector cells in fibrosis, and this mesenchymal cell phenotype will be discussed in more detail in **Section 1.3.2.3**. Interestingly, recent evidence suggests that fibrotic foci are, in fact, interconnected, forming a continuous fibrotic reticulum which suggests that individual foci may not arise from discrete sites of lung injury (Cool *et al.*, 2006). In lung fibrosis, it has been postulated that persistent and/or repetitive injurious stimuli result in a dysregulated wound healing response, characterised by impaired alveolar re-epithelialisation. Thereafter, persistent myofibroblast accumulation and activation contributes to the excessive deposition of ECM in the interstitium, characteristic of pulmonary fibrosis.

Prevailing hypotheses regarding the pathogenesis of fibroproliferative lung disease have focused on dysregulated interactions between epithelial and mesenchymal cells (Selman *et al.*, 2002). Moreover, there is increasing evidence that this defective crosstalk may be amplified by the immune response to injury (Wynn, 2004). These concepts will now be discussed in more detail.

1.3.2.1. *Dysregulated epithelial – mesenchymal interactions in fibroproliferative disease*

Contemporary hypotheses propose that IPF arises as a result of a highly aberrant wound healing response in susceptible individuals following repetitive or persistent epithelial injury; it has been postulated that that IPF is an “epithelial-fibroblastic disease” in which fibroproliferative pathology is preceded by AEC injury and activation, with fibrotic foci representing the primary sites of injury and aberrant repair (Selman *et al.*, 2002). The molecular mechanisms underlying the development of these foci, however, remain unclear – recent evidence suggests roles for the local proliferation, activation and differentiation into myofibroblasts from resident lung fibroblasts; recruitment of circulating fibrocytes; epithelial-

mesenchymal transition and endothelial-mesenchymal transition. The derivation of (myo)fibroblasts will be discussed in greater detail in **Section 1.3.2.3.**, but in all of these processes, a prominent role has been suggested for the overlying reactive and hyperplastic epithelium, resulting in a final common pathology characterized by the excessive and disordered deposition of ECM proteins with the destruction of alveolar-capillary units and the formation of cystic fibrotic spaces. The role of the epithelium and the mesenchyme in the pathogenesis of lung fibrosis will now be discussed in turn.

1.3.2.2. *The epithelium in IPF*

As alluded to earlier, an early feature of pulmonary fibrosis in humans is a change in the phenotype of the AEC, suggesting that AEC injury is a critical step in the pathogenesis of pulmonary fibrosis (Chilosi *et al.*, 2002; Kasper *et al.*, 1996). These changes include apoptosis (Kuwano *et al.*, 1996; Plataki *et al.*, 2005), regenerative hyperplasia (Corrin *et al.*, 1985), bronchiolarization (Kawanami *et al.*, 1982; Sutinen *et al.*, 1980) and proliferation (Katzenstein, 1985). In IPF, epithelial cell apoptosis is a well-recognised histological finding (Barbas-Filho *et al.*, 2001; Kuwano *et al.*, 1996; Maeyama *et al.*, 2001; Plataki *et al.*, 2005; Uhal *et al.*, 1998). The mechanisms underlying this apoptosis, however, are unclear, though numerous mediators have been proposed, including TGF- β (Lee *et al.*, 2004); angiotensin II (Marshall *et al.*, 2004; Wang *et al.*, 1999); reactive oxygen species (Waghray *et al.*, 2005) and Fas-signalling pathways (Maeyama *et al.*, 2001). More recently, the alveolar epithelium of patients with IPF has been shown to express markers of endoplasmic reticulum stress and the unfolded protein response - activation of these pathways may result from altered surfactant protein processing or chronic herpesvirus infection (Korfei *et al.*, 2008; Lawson *et al.*, 2008).

The persistent apoptosis and dysregulated proliferation of epithelial cells impairs adequate epithelial reconstitution following lung injury. A reconstituted epithelium appears to serve a homeostatic function that may be necessary to suppress the excessive fibroblast activation characteristic of an aberrant wound healing response. Prostaglandin E₂ (PGE₂), a cyclooxygenase (COX)-dependent arachidonic acid metabolite, is the major prostanoid produced in the lung. It is secreted by intact epithelial cells as well as fibroblasts (Maher *et al.*, 2010a). Its anti-fibrotic functions have been demonstrated both *in vitro* (Goldstein *et al.*, 1982; Kolodsick *et al.*, 2003; Lama *et al.*, 2002) and *in vivo* (Keerthisingam *et al.*, 2001) and levels of PGE₂ have been reported to be reduced in IPF lung (Borok *et al.*,

1991). Furthermore, PGE₂ has recently been implicated as playing a central role in promoting the 'apoptosis paradox' of IPF in which excessive epithelial cell apoptosis and (myo)fibroblast resistance to apoptosis contribute to dysregulated epithelial-mesenchymal interaction (Maher *et al.*, 2010b).

As alluded to above, epithelial cell proliferation and regeneration following injury is critical to normal wound healing. In this regard, the anti-fibrotic activity of epithelial mitogens such as keratinocyte growth factor (KGF) (Aguilar *et al.*, 2009) and hepatocyte growth factor (HGF) (Dohi *et al.*, 2000) *in vivo* suggest that the promotion of appropriate re-epithelialisation may have therapeutic benefits in IPF. However, because IPF patients are at increased risk of developing lung malignancies (Ozawa *et al.*, 2009), such a strategy would require further exploration before human trials are deemed ethical.

Impaired epithelial reconstitution may further contribute to fibrogenesis through the generation of pro-fibrotic cytokines such as TGF- β (Xu *et al.*, 2003) and TNF- α (Nash *et al.*, 1993). TGF- β is regarded as a central mediator of pulmonary fibrosis in both murine models of fibrosis and human disease (Giri *et al.*, 1993; Khalil *et al.*, 1989; Khalil *et al.*, 1991). Its mechanisms of action are multitude and include the promotion of fibroblast to myofibroblast differentiation (Chambers *et al.*, 2003; Subramanian *et al.*, 2004), epithelial-mesenchymal transition (EMT) (Willis *et al.*, 2005) and the inhibition of myofibroblast apoptosis (Zhang *et al.*, 1999). TGF- β is a potent inducer of collagen deposition, promoting both its synthesis (Raghow *et al.*, 1987) and inhibiting its degradation (McAnulty *et al.*, 1991; Overall *et al.*, 1989). Furthermore, TGF- β is capable of upregulating the expression of a number of other pro-fibrotic mediators in fibroblasts, such as connective tissue growth factor (CTGF) (Igarashi *et al.*, 1993). Interestingly, there is evidence of an overlapping relationship between the actions of TGF- β and TNF- α in the pathogenesis of lung fibrosis. Attenuation of experimentally-induced fibrosis in mice, using genetic or pharmacological tools to interfere with TNF- α signalling, is associated with reduced TGF- β expression (Liu *et al.*, 1998; Phan *et al.*, 1992; Piguet *et al.*, 1994). Moreover, local over-expression of TNF- α in mice results in a patchy parenchymal fibrosis which is associated with increased TGF- β expression (Sime *et al.*, 1998). *In vitro* studies have also highlighted the pro-fibrotic inter-relationship between TGF- β and TNF- α , demonstrating that TNF- α increases active TGF- β bioavailability both by increasing its transcription and mRNA stability (Sullivan *et al.*, 2009; Sullivan *et al.*, 2005).

Finally recent studies have demonstrated the importance of danger signals, derived from an injured epithelium, in promoting lung fibrosis in an interleukin (IL)-1 β -dependent / inflammasome dependent manner (Gasse *et al.*, 2009; Riteau *et al.*, 2010). These non-antigenic danger signals, in addition to microbial products, alert the immune system to tissue stress or injury, and such detection is increasingly recognised as crucial to maintaining host integrity (Matzinger, 1994). It has previously been suggested that TNF- α be regarded as an endogenous danger signal (Efron *et al.*, 2005), particularly in the context of its well documented capacity to modulate the innate and adaptive arms of the immune system (Kollias *et al.*, 1999). Although immune cells, such as alveolar macrophages have traditionally been regarded as the predominant source of TNF- α in the lung (Vassalli, 1992), injured epithelial cells are recognised as an important source of this master cytokine in fibroproliferative lung disease (Nash *et al.*, 1993; Oikonomou *et al.*, 2006; Piguet *et al.*, 1993). Increased epithelial expression of TNF- α following epithelial injury may therefore be regarded as an initial danger signal to the immune system, and the role of TNF- α in the pathogenesis of lung fibrosis will be discussed in more detail in **Section 1.8.2.**

1.3.2.3. *The (myo)fibroblast in IPF*

The myofibroblast has long been regarded as the major cell involved in normal wound healing, and as the key effector cell in fibrogenesis. Myofibroblasts have characteristics intermediate between fibroblasts and smooth muscle cells (Gabbiani, 2003). They are highly synthetic for collagen and other ECM components, and express α -smooth muscle actin (α -SMA) – the extent of such expression correlates with the increased contractility of this cell phenotype (Hinz *et al.*, 2001), though the underlying mechanism of increased contractility is, as yet, not fully understood. Early studies observed the transient presence of the myofibroblast within granulation tissue, a cell phenotype exhibiting smooth muscle cell-like features including intracellular bundles of microfilaments and significant amounts of endoplasmic reticulum (Desmouliere *et al.*, 2005). Other studies demonstrated the importance of myofibroblasts in a number of pathological conditions characterised by aberrant wound healing and excessive extracellular matrix deposition, such as fibrotic conditions of the heart (Brown *et al.*, 2005), liver (Desmouliere *et al.*, 2003) and lung (Adler *et al.*, 1989).

The presence of myofibroblasts in IPF lung, and in fibrotic lesions of animal models of fibrosis (Adler *et al.*, 1989; Mitchell *et al.*, 1989; Pache *et al.*, 1998), supports the

notion that this mesenchymal phenotype contributes to the progression of fibrosis. Myofibroblasts have an increased synthetic capacity for collagen and other ECM components known to be associated with active disease such as fibronectin, vitronectin and laminin (Hetzel *et al.*, 2005; Kuhn *et al.*, 1991). Their presence in fibrotic lesions correlates with the development of active fibrosis in animal models, and their persistence and localisation to fibrotic foci in human disease is associated with disease progression (Kuhn *et al.*, 1991; Zhang *et al.*, 1994b).

As well as demonstrating an increased synthetic capacity for collagen, myofibroblasts isolated from IPF lungs exhibit an enhanced migratory phenotype (Suganuma *et al.*, 1995) and produce more pro-fibrotic mediators (Moodley *et al.*, 2003) when compared to mesenchymal cells isolated from healthy lungs. In addition, they appear more resistant to apoptosis (Moodley *et al.*, 2004; Ramos *et al.*, 2001) – failure of apoptosis following appropriate wound healing results in the persistence of this highly activated mesenchymal cell at the site of injury. In fact, TGF- β , whilst appearing to promote apoptosis of epithelial cells (Lee *et al.*, 2004), promotes an anti-apoptotic phenotype in fibroblasts (Horowitz *et al.*, 2006; Zhang *et al.*, 1999).

The myofibroblast itself may also induce epithelial apoptosis through paracrine signalling via extracellular hydrogen peroxide (Uhal *et al.*, 1995; Waghray *et al.*, 2005), whilst autocrine signalling, mediated by growth factors, can activate cell survival pathways (Horowitz *et al.*, 2004). The autocrine induction of TGF- β by myofibroblasts is mirrored by the decreased production of epithelial mitogens such as HGF and KGF (Marchand-Adam *et al.*, 2003; Marchand-Adam *et al.*, 2005), as well as anti-fibrotic mediators such as PGE₂ (Keerthisingam *et al.*, 2001).

Classically, myofibroblasts in pulmonary fibrosis were thought to be derived from the local proliferation and subsequent differentiation of resident fibroblasts in the context of a highly pro-fibrotic cytokine milieu (Phan, 2002; Zhang *et al.*, 1994b). However, this notion has recently been challenged, and it is now recognised that the key effector cell of fibrosis may have a number of other cellular sources. The concept of epithelial-mesenchymal transition (EMT) has been recognised as contributing to functional myofibroblasts in a number of other human fibrotic diseases (Carew *et al.*, 2012), and there is some evidence to support a role for EMT in IPF. During EMT, epithelial cells lose their characteristic markers (e.g. E-cadherin and zona occludens-1) and acquire mesenchymal markers (e.g. fibroblast-specific protein-1 and α -SMA) (Grunert *et al.*, 2003). AECs *in vitro* undergo EMT following prolonged

exposure to major fibrogenic mediators such as TGF- β when cultured on a provisional wound matrix – this process is further enhanced in the presence of TNF- α (Willis *et al.*, 2005). Lineage tracing studies have also provided strong support for EMT as a potential source of myofibroblasts during lung fibrogenesis (Kim *et al.*, 2006b; Kim *et al.*, 2009). The observation that cells in IPF biopsy samples co-express epithelial and mesenchymal markers (Kim *et al.*, 2009) support the notion of EMT in human disease although it must be acknowledged that this has not been a universal finding (Yamada *et al.*, 2008). The molecular pathways underlying the development of EMT are proving to be a fertile area of research, with current evidence suggesting roles for Wnt and Notch signalling pathways (Aoyagi-Ikeda *et al.*, 2010; Chilos *et al.*, 2003; Konigshoff *et al.*, 2009).

A second hypothesis regarding the origin of (myo)fibroblasts in lung fibrosis proposes that these cells may be derived from circulating fibrocytes (Lama *et al.*, 2006). Fibrocytes were originally identified as collagen I + /CD34+/CD45RO+ cells that are likely derived from hematopoietic stem cells (Bucala *et al.*, 1994). Support for a pathogenic role for fibrocytes in lung fibrosis has been provided from studies showing that blockade of fibrocyte recruitment is protective following experimental lung injury in rodents (Moore *et al.*, 2005; Phillips *et al.*, 2004). Specific chemokine receptor/chemokine ligand biological axes are critical to the recruitment of fibrocytes to sites of tissue injury and repair, where they may contribute to the propagation of the fibrotic response. A major role has been identified for the CXCR4/CXCL12 axis in this respect (Phillips *et al.*, 2004) although several chemokines have been shown to be capable of recruiting fibrocytes *in vivo*. Whether fibrocyte-derived fibroblasts are capable of differentiating into fully activated myofibroblasts, especially in patients with IPF, remains the subject of an interesting debate, although recent evidence suggests that ~10% of fibrocytes express α -SMA in the bleomycin model (Mehrad *et al.*, 2009). CXCR4/fibrocyte/mesenchymal marker co-expression studies further support the notion that circulating fibrocytes may contribute to the expansion of the fibroblast/myofibroblast population in IPF (Andersson-Sjoland *et al.*, 2008). Finally, a recent report has shown that a >5% blood fibrocyte count is associated with poor survival in IPF (Moeller *et al.*, 2009). In addition to the above cellular sources, there is very recent experimental evidence that lung capillary endothelial cells may also give rise to fibroblasts through endothelial–mesenchymal transition in a bleomycin-induced lung fibrosis model (Hashimoto *et al.*, 2010).

As has been alluded to earlier in this section, dysregulated epithelial-mesenchymal interactions following lung injury, driven by mediators derived from these two cellular compartments, result in persistent activation of fibroblasts. However, there remains good evidence that these pro-fibrotic interactions may be further amplified by concomitant inflammatory and immune responses. Exciting new research has highlighted the role of danger signals, reflecting cellular injury, in potentiating the innate and adaptive immune response to epithelial injury. The nature and role of such cells and mediators in activating the immune response and thus enhancing the fibrotic response to injury will be discussed in **Section 1.5**. However, in the first instance, a brief overview of the role of the inflammatory and immune system in the pathogenesis of IPF will now be provided.

1.3.3. The role of inflammation in the pathogenesis of fibrosis

The role of inflammation *per se* in the pathogenesis of pulmonary fibrosis is controversial and continues to be the subject of much debate. For many years, chronic inflammation of the epithelial and vascular compartments was felt to be the dominant mechanism driving fibrogenesis, and IPF was regarded as primarily a chronic inflammatory condition. The failure of anti-inflammatory and immunosuppressive therapy to have any impact on the mortality of patients with IPF, together with the lack of any significant inflammatory histology on IPF lung specimens, prompted a paradigm shift in the postulated mechanisms involved in the pathogenesis of fibroproliferative lung disease. This has been supported by observations from animal models of fibrosis, where significant bleomycin-induced inflammation in $\alpha\text{v}\beta 6$ integrin knockout mice (which are unable to activate TGF- β effectively) does not progress to fibrosis (Munger *et al.*, 1999). Nonetheless, leading investigators continue to argue that inflammation does play a role in the early initiating (sub-clinical) stages of IPF (Strieter, 2008), and the evidence in support of this position will now be discussed.

The lung occupies a unique position between the host and the external environment, with the alveolar space being separated from the vasculature by a barrier of only 4–8 μm , the alveolar-capillary membrane (ACM). Clearly, this anatomical structure is ideal for gas exchange, but leads to increasing host vulnerability to environmental insults and subsequent injury. The lung must be able to initiate a rapid (innate) host response to any offending insults, while maintaining its ability to facilitate effective gas exchange. Following lung injury, the complex interplay between humoral, cellular and ECM networks, in a sequential and

overlapping manner, is designed to restore baseline integrity to the ACM. Initially, haemorrhage and extravasation of plasma into the alveolar space results in activation of both the intrinsic and extrinsic coagulation cascade resulting in fibrin generation and the initiation of a provisional wound matrix. Concomitant platelet activation leads to the generation of numerous chemokines and cytokines, whose function include to promote the influx of appropriate inflammatory and mesenchymal cells and to elaborate the provisional matrix in such a manner as to facilitate the repair process. The initial influx of neutrophils is followed by the appearance of mononuclear cells, orchestrated to a degree by neutrophil-derived mediators (Keane MP, 2005). This second wave of inflammatory cell influx is crucial to the generation of further inflammatory and reparative mediators responsible for maturation of the ECM (Sibille *et al.*, 1990). The immature provisional matrix is primarily composed of fibrin, fibronectin and a preponderance of type III collagen over type I, in contrast to the mature ECM, whose predominant collagen is type I (Keane MP, 2005). In instances where the basement membrane (BM) of the ACM has not been breached, resorption of the mature ECM allows appropriate re-epithelialization (facilitated by contraction of myofibroblasts) and re-endothelialization of the ACM, with restoration of a functioning alveolar unit (Wallace *et al.*, 2007). Subsequent apoptosis of mesenchymal cells ensures the cessation of matrix deposition with minimal residual tissue damage.

It has been argued that inflammation-induced loss of ACM BM integrity represents “a point of no return” in fibroproliferative lung disease, as adequate re-epithelialization and re-endothelialization cannot occur on a persistently denuded BM (Strieter, 2008). Due to a combination of host and environmental factors, inappropriate proliferation of type II pneumocytes, in concert with persistent activation of (myo)fibroblasts and continuing deposition of mature ECM, leads to the histological appearance of UIP. This loss of integrity of the BM is consistently seen in ultrastructural analysis of UIP, as is the intra-alveolar deposition of ECM associated with myofibroblast accumulation (Basset *et al.*, 1986; Corrin *et al.*, 1985; Wallace *et al.*, 2007).

Importantly, UIP, whilst characteristic of IPF in the correct clinical context, can also represent end-stage pathology in a number of inflammatory conditions of the lung, including hypersensitivity pneumonitis, as well the pulmonary manifestation of chronic systemic inflammatory conditions such as rheumatoid arthritis and systemic sclerosis (Wallace *et al.*, 2007). These conditions are characterised by the

persistence of an injurious insult, be it an exogenous or endogenous antigen. These observations suggest that UIP may occur in the context of preceding inflammation, and that our current snapshot view of this disease, almost always at late to end-stage at presentation (King *et al.*, 2001), precludes the detection of any antecedent inflammation. In fact, lung inflammation is seen in unaffected family members of patients with an autosomal dominant form of IPF, though it is unclear if these individuals will go on to develop a UIP lung histology (Bitterman *et al.*, 1986). Moreover, even within IPF lung, the more inflammatory histology of NSIP is seen in 26% of patients (Flaherty *et al.*, 2001), suggesting that the two disease profiles may share a common origin. Indeed, no differences are observed in the global gene expression profiles of these conditions (Rosas *et al.*, 2007). Recent gene microarray studies have demonstrated that, in addition to the expected increase in gene expression of proteins associated with extracellular matrix turnover, expression of genes traditionally associated with inflammatory process such as cytokines and chemokines is increased in IPF (Zuo *et al.*, 2002). In an attempt to bring together two extremes of opinion, it has been suggested that while inflammation is necessary for fibrosis to develop, it is not sufficient in itself. Transient overexpression of the pro-inflammatory cytokine IL-1 β leads to an acute inflammatory response which progresses to fibrosis in the context of sustained TGF- β expression (Kolb *et al.*, 2001) – it has been proposed that the initial inflammatory response following tissue injury induces dysregulation of the epithelial-mesenchymal interaction resulting in failure of re-epithelialisation and persistence of myofibroblasts driven by cytokines such as TGF- β (Zhang *et al.*, 1999).

More recently, the potential role of the adaptive immune response in the pathogenesis of IPF has been highlighted. In particular, a cytokine milieu dominated by T-helper 2 (Th2), or Type-2 (T-2), cytokines, such as IL-4 and IL-13, following injury is widely regarded as being pro-fibrotic (Wynn, 2004). Crucial to polarisation of the immune response towards a T-2 phenotype are activated dendritic cells (DCs), through their interactions with naïve CD4 T-cells and memory Th2 cells. Activation of immature DCs had previously been thought to be related to their ability to sense the presence of foreign microbial products, allowing them to present foreign antigen, following intracellular processing, to naïve T-cells. However, it is increasingly appreciated that such activation may be induced by non-microbial danger signals, reflecting cellular injury or disruption to tissue architecture (Johnson *et al.*, 2002; Matzinger, 1994; Shi *et al.*, 2003), in addition to pathogen-associated molecular patterns (PAMPs) (Pulendran, 2004). Thymic stromal lymphopoietin

(TSLP) is a recently discovered IL-7 like cytokine that has emerged as a key regulator of inflammatory responses characterised by increased expression of T-2 cytokines, via its activation of immature DCs. However, its role in the pathogenesis of non-allergen driven lung diseases characterised by increased T-2 cytokine expression has not been explored. In this regard, the work presented in this thesis will attempt to address this issue, highlighting the potential role of TSLP as a T-2 polarising signal relayed from the mesenchyme to immune cells, reflecting epithelial injury.

These concepts will now be explored in more detail, beginning with a more extensive description of TSLP, its signalling pathways and its activation of immature DCs. The importance of mature DCs in the pathogenesis of IPF, in particular highlighting the importance of their activation by danger signals reflecting epithelial injury, will then be addressed. Thereafter, a brief summary of evidence supporting the notion that increased T-2 cytokine expression can amplify the fibroproliferative response to injury will be provided. A major part of the work contributing to this thesis has explored the importance of TNF- α in mediating TSLP expression, and this introduction will conclude by highlighting the importance of this master cytokine in mediating the defective epithelial-mesenchymal crosstalk, considered crucial in the pathogenesis of lung fibrosis.

1.4 Thymic stromal lymphopoietin (TSLP)

Thymic stromal lymphopoietin (TSLP) is a novel IL-7-like cytokine that has recently been shown to be a critical regulator of atopic inflammatory responses in the lung characterised by increased expression of T-2 cytokines. The work in this thesis evaluates the role of TSLP in fibrotic lung disease. A more extensive description of TSLP, its expression and signalling pathways, and its effect on promoting the differentiation of naïve T cells into Th2 cells will now be discussed.

1.4.1. Structure

TSLP is a type I cytokine that belongs to the IL-2 cytokine family; this family includes IL-2, IL-4, IL-7 and IL-13 (Leonard, 2001). Of these members, TSLP is regarded as being most similar in function to IL-7. It was initially identified as a factor in the supernatants of a thymic stromal cell line that was capable of supporting the growth and development of murine fetal liver and adult bone marrow derived B cell progenitors (Friend *et al.*, 1994; Sims *et al.*, 2000), much like IL-7.

Conditioned medium from this cell line supported the long term growth of the NAG8/7 pre-B cell line and enhanced the proliferation of thymocytes to sub-optimal concentrations of anti-CD3 antibodies *in vitro*. However, unlike IL-7, TSLP is capable of promoting B cell development to a more mature B220+ / IgM+ stage, compared to a B220+ / IgM- stage (Levin *et al.*, 1999). In fact, TSLP appears to influence B cell progenitor cells to varying degrees, dependent upon their origin – bone marrow derived pro-B cells, unlike fetal liver derived pro-B cells, must develop to a pre-B cell stage before they can respond to TSLP, despite expressing the TSLP receptor complex (Vosshenrich *et al.*, 2004).

Characterisation of this murine factor revealed a four-helix bundle 140 amino acid cytokine (Sims *et al.*, 2000), and a human orthologue of TSLP was identified thereafter (Quentmeier *et al.*, 2001). Human TSLP exhibits a similar four-helix structure with two N-glycosylation sites and six cysteine residues. Interestingly, the human TSLP gene is located in close proximity to a gene cluster encoding T-2 cytokines on chromosome 5 (Quentmeier *et al.*, 2001). Although human TSLP and murine TSLP share poor sequence homology of 43%, they are both expressed by similar cells and seem to exert similar biological functions (Liu *et al.*, 2007b).

1.4.2. TSLP receptor complex and signalling pathway

The TSLP receptor complex (TSLPR) is a heterodimeric receptor consisting of a TSLP α -binding chain (referred to as TSLPR α) and the IL-7R α chain (Park *et al.*, 2000). The former is a member of the haematopoietin receptor family and binds to TSLP with low affinity. It is an atypical type I cytokine receptor, with 24% homology to the common γ c receptor chain, but has significant differences to other type I cytokine receptor chains, both in its intracellular and extracellular regions – intracellularly, for instance, while it contains a conserved cytoplasmic Box 1 domain, it lacks the conserved Box 2 domain, both of which regulate Janus protein tyrosine kinase (JAK) binding (Liu *et al.*, 2007b). To date, expression of the human TSLP receptor complex has been identified in activated CD4+ cells, myeloid DCs and mast cells (Omori *et al.*, 2007; Soumelis *et al.*, 2002). More recently, structural cells have also been demonstrated to be capable of responding to TSLP (Semlali *et al.*, 2010; Shan *et al.*, 2010) and TSLPR expression has been documented in human skin, kidney, liver and skeletal muscle (Tonozyuka *et al.*, 2001).

Dimerization with the IL-7R α chain allows high affinity binding and the initiation of downstream cell signalling pathways with functional effects (Park *et al.*, 2000;

Reche *et al.*, 2001). In particular, ligand-induced cross-linking of TSLPR α and IL-7R α results in functional activation of signal transducer and activator of transcription (STAT) 5 and 3 (Isaksen *et al.*, 1999; Quentmeier *et al.*, 2001; Wohlmann *et al.*, 2010) (**Figure 1.2**). Ordinarily, cytokine receptor-induced aggregation induces JAK transphosphorylation and subsequent phosphorylation of target tyrosine residues of the receptor. These then act as docking sites for -SH2 domain containing signalling molecules including STATs. STAT binding results in JAK-induced phosphorylation of tyrosine residues, inducing conformational shifts which permit dimerization and subsequent translocation to the nucleus to initiate transcription of target genes. Certainly, the IL-7 receptor complex, comprising the common γ_c chain and IL-7R α , recruits JAK3 via the IL-7R α Box 1 domain, leading to STAT5 activation, and subsequent target gene regulation (Hofmeister *et al.*, 1999). However, activated JAK kinases do not exhibit specificity for particular STATs, as different receptors may activate common STATs, despite engaging with different JAKs (Kohlhuber *et al.*, 1997). In fact specificity for STAT activation seems to be determined by the docking sites present on the receptor chains themselves. In addition, STATs may also be phosphorylated in a JAK-independent manner by receptor kinases directly, as in the case of the epidermal growth factor receptor (David *et al.*, 1996), or by non-receptor associated kinases, such as Src family kinases (Chaturvedi *et al.*, 1998).

Initial studies suggested that TSLPR ligation appeared to drive STAT5 and 3 activation without involving any known JAKs (Isaksen *et al.*, 1999; Levin *et al.*, 1999), despite the presence in both chains of conserved residues of typical Box1 motifs – these sequences mediate the association of type I cytokine receptor chains with JAKs (Tanner *et al.*, 1995). Mutations in the Box 1 domains from either chain abrogates TSLP-induced STAT activity (Isaksen *et al.*, 2002) suggesting an involvement of JAK downstream of receptor ligation. However, TSLP did not appear to induce any JAK phosphorylation, despite the induction of functional STAT activity (Levin *et al.*, 1999). Moreover, reconstitution studies demonstrated that expression of dominant-negative forms of JAK1 and JAK2 were unable to block TSLP-induced STAT5 phosphorylation, in contrast to IL-7 signalling (Isaksen *et al.*, 1999). However, partial inhibition of TSLP-induced STAT5 activation was observed in the presence of a dominant-negative version of Tec, a cytoplasmic Src-related tyrosine kinase, suggesting a role for this kinase family upstream of STAT5 (Isaksen *et al.*, 1999). Further complexity to the TSLP signalling pathway is demonstrated by apparent uncoupling of STAT5 activation and TSLP-induced proliferation in pre B

cell lines - inhibition of the Src family kinases blocks TSLP-induced proliferation but has no effect on STAT5 activation (Isaksen *et al.*, 2002).

However, more recent studies have attempted to reconcile these unexpected findings. Earlier work evaluated JAK-STAT signalling pathways in murine pre-B cell lines in contrast to studies demonstrating a requirement for JAK1 and JAK2 in TSLP-induced STAT activation and downstream cellular effects in murine primary T cells (Rochman *et al.*, 2010). These observations are supported by studies demonstrating a clear requirement for JAK1 and JAK2 in mediating STAT phosphorylation downstream of human TSLP receptor ligation (Wohlmann *et al.*, 2010). These latter differences may be explained by differences in downstream signalling pathways between mice and humans, supported by the fact that murine and human TSLPR share only 39% sequence homology – in particular, the highly conserved Box1 domain is located in different positions in these species.

In summary, the mechanisms involved in TSLP signalling via the heterodimeric TSLPR α / IL-7R α complex remain unclear. The unexpected findings that TSLP-induced STAT phosphorylation in the absence of JAK phosphorylation have recently been addressed, though additional studies are required to fully delineate the signalling pathways downstream of TSLP receptor ligation and it is possible that unidentified kinases upstream of STAT are also involved in signal transduction.

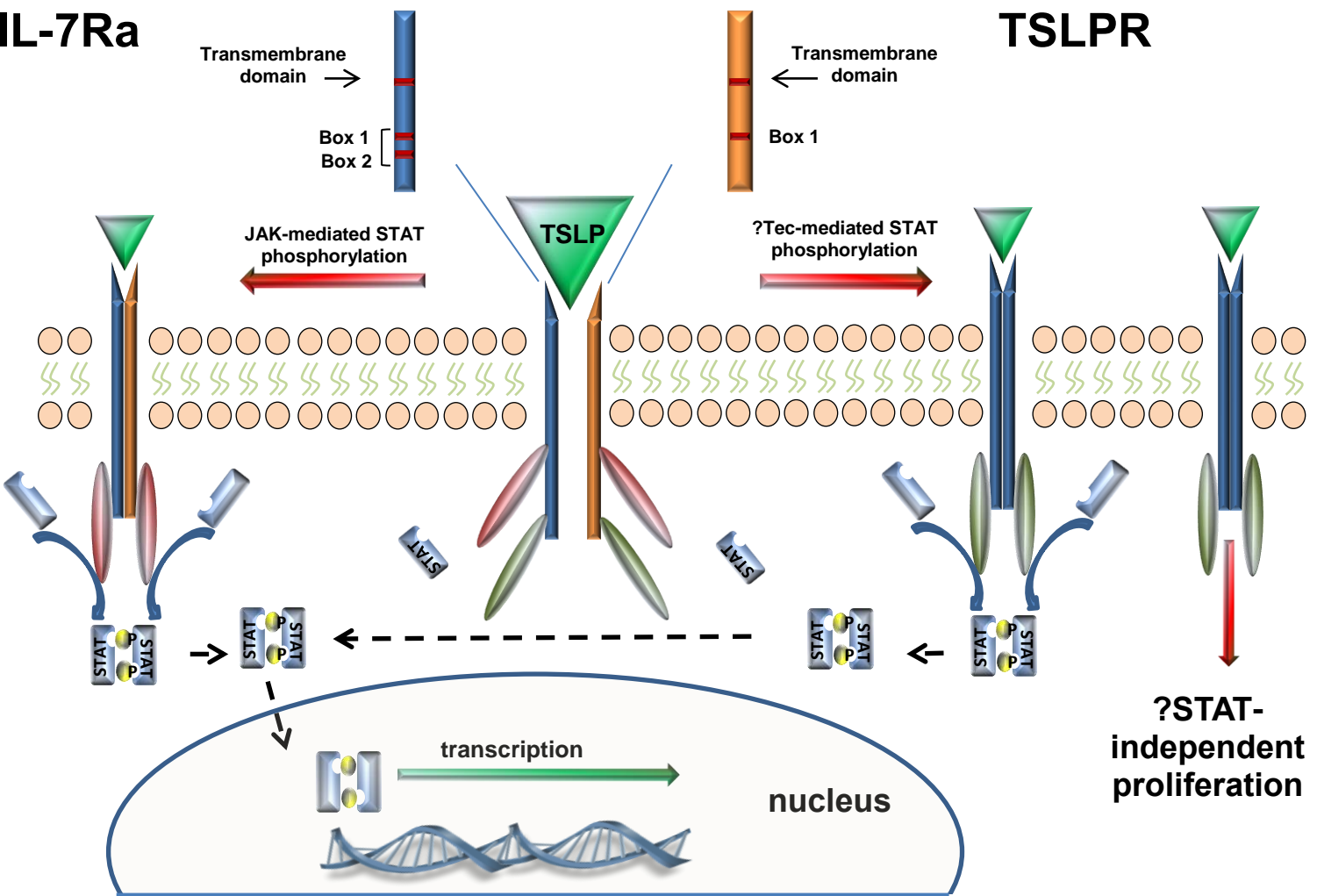


Figure 1.2. Structure and signal transduction of the TSLP receptor. Dimerization of the TSLPR and IL-7Ra chains permits high-affinity binding of TSLP. Downstream STAT signalling may be initiated in a JAK-dependent or –independent manner which may reflect inter-species variation. TSLP-induced proliferation may also be uncoupled from STAT phosphorylation.

1.4.3. TSLP expression in pulmonary cells

TSLP expression has been most extensively studied in the context of archetypal T-2 cytokine dominated diseases, such as atopic asthma and atopic dermatitis, with much of our understanding of the mechanisms involved in regulating its expression *in vivo* coming from studies of animal models of these conditions.

In humans, baseline TSLP mRNA transcripts have been detected for a number of key pulmonary cells, including bronchial and small airway epithelial cells, smooth muscle cells, fibroblasts and mast cells (Soumelis *et al.*, 2002). Confirmation of expression at the protein level has been confirmed for epithelial cells (Lee *et al.*, 2007a), but not for fibroblasts. *In vitro* work has identified a number of mediators capable of inducing TSLP expression by lung epithelial and smooth muscle cells, and these are outlined in **Table 1.3**. However, the signal transduction systems underlying induction of TSLP expression remain unclear and are likely to vary between cell types. Recognised signalling pathways leading to TSLP production will now be discussed in more detail.

1.4.3.1. Role of mitogen-activated protein kinase pathways in regulating TSLP expression

Activation of mitogen-activated protein kinase (MAPK) signalling represents a major signal transduction pathway responsible for relaying extracellular stimuli allowing regulation of intracellular function. The MAPK family consists of 3 major sub-groups of highly conserved, eukaryote specific serine-threonine kinases, including the c-Jun amino-terminal kinases (JNK 1/2/3), extracellular signal-regulated kinases (ERK 1/2) and p38 kinases. Briefly, the MAP kinases are sequentially phosphorylated by upstream kinases (Kyriakis *et al.*, 2001), beginning with MAPK kinase kinases (MAPKKKs or MEKKs). These then activate downstream MAPK kinases (MAPKKs or MEKs) by phosphorylation of specific serine and threonine residues (Widmann *et al.*, 1999). MEKs in turn phosphorylate MAPKs facilitating MAPK translocation to the nucleus where they can interact with transcription factors and histone proteins to regulate transcription of target genes (Treisman, 1996).

The ERK1/2 pathway is important in mediating a number of responses in fibroblasts, such as proliferation and collagen production (Yoshida *et al.*, 2002). This pathway has also been shown to mediate TNF- α induced TSLP protein production by human airway smooth muscle cells (HASMCs) – pharmacological inhibition of ERK1/2 resulted in a significant inhibition of IL-1 β and TNF- α induced TSLP expression

Stimulant	Cell type	TSLP expression		References
		mRNA	protein	
Poly I:C	Primary small airway epithelial cells	+	+	(Allakhverdi <i>et al.</i> , 2007b)
Bacterial peptidoglycan		+	+	
IL-4	NHBE	+	-	(Kato <i>et al.</i> , 2007)
IL-13		+	-	
IL-4 + TNF- α	NHBE	+	+	(Kato <i>et al.</i> , 2007)
IL-13 + TNF- α		+	+	
IL-1 β	NHBE	+	+	(Lee <i>et al.</i> , 2007a)
TNF- α	A549	+	+	
IL-1 β	Primary airway smooth muscle cells	+	+	(Zhang <i>et al.</i> , 2007)
TNF- α		+	+	
TLRL 2/3/8/9	NHBE	+	-	(Lee <i>et al.</i> , 2007a; Zhang <i>et al.</i> , 2007)
TLRL 3 + IL-4	NHBE	+	+	

+/-: increased / unchanged expression
 NHBE: normal human bronchial epithelial cells
 TLRL: toll like receptor ligand

Table 1.3.

Summary of known inducers of TSLP expression in pulmonary cells

(Zhang *et al.*, 2007), suggesting that TSLP expression in these cells is controlled via MAPK pathways.

The primary role of p38 has been considered to be in mediating inflammatory responses in disease settings (Schindler *et al.*, 2007), and this kinase has been implicated in mediating the airway inflammation found in asthma, COPD and cystic fibrosis (de Boer *et al.*, 2007; Konstan *et al.*, 2002; Pelaia *et al.*, 2005). Pharmacological inhibition of the p38 pathway suppresses TNF- α induced TSLP secretion by HASMCs (Zhang *et al.*, 2007). The potential importance of this member of the MAPK family in mediating TSLP expression is further highlighted by studies performed in normal human bronchial epithelial cells (NHBEs). Respiratory syncytial virus (RSV) infection is a well-documented trigger of asthmatic exacerbations and can upregulate TSLP expression by NHBEs. Such induction is attenuated by inhibition of the p38 pathway (Tu *et al.*, 2007).

Three isoforms of JNK are recognized; JNK1 and JNK2 are broadly expressed in many tissues, whereas JNK3 is exclusively expressed by neurons, cardiac myocytes and testes. Therefore, only JNK1 and JNK2 are relevant to respiratory pathobiology (Bennett, 2006). JNK is the enzyme responsible for phosphorylating critical serine residues in the N-terminus of c-Jun, a component of AP-1. TSLP expression in NHBEs is upregulated following infection with rhinovirus and respiratory syncytial virus, and this effect is attenuated by pre-treatment of cells with inhibitors of the JNK pathway, implicating the JNK pathway in mediating TSLP expression in this cell type (Tu *et al.*, 2007).

1.4.3.2. The role of AP-1 in mediating TSLP expression

The transcription factor, AP-1, is a heterodimeric protein complex composed of c-Jun and c-Fos components. Regulation of AP-1 activity is achieved via a number of mechanisms including changes in transcription of genes encoding AP-1 subunits, control of the stability of their mRNAs and specific interactions between AP-1 proteins and other transcription factors and cofactors [reviewed in (Hess *et al.*, 2004)]. Post-translational control has been most extensively documented for MAPKs; differential phosphorylation of c-Jun and c-Fos is essential for AP-1 directed transcription of target genes and is achieved primarily by activation of kinases of the MAPK family of enzymes, in particular, JNK. With respect to the present studies, the importance of this transcription factor in mediating the expression of TSLP is suggested by the identification of a single nucleotide

polymorphism (SNP) in the promoter region of a long splice variant of the human *Ts/p* gene in human bronchial epithelial cells, facilitating the creation of a functional AP-1 binding site (Harada *et al.*, 2009). Furthermore, a putative AP-1 binding site has been identified in the *Ts/p* gene promoter region of a lung epithelial cell line and mutations in this motif result in a reduction in IL-1 β induced promoter activity (Lee *et al.*, 2007a). In human airway smooth muscle cells, transfection with a TSLP promoter plasmid harboring mutations in AP-1 binding sites abrogated TSLP promoter activity following TNF- α exposure (Redhu *et al.*, 2011), further supporting a role for this transcription factor in mediating TSLP expression.

1.4.3.3. *Role of the NF κ B in regulating TSLP expression*

The transcription factor NF κ B is activated by numerous stimuli, and once activated, participates in the regulation of various target genes in different cells to exert its biological functions.

Briefly, the NF κ B family of transcription factors comprises a group of dimeric factors that are involved in the regulation of a huge range of inflammatory responses. NF κ B proteins are retained within the cytoplasm bound to cellular I κ B proteins which mask the former's nuclear location sequence. Liberation of NF κ B from this ternary complex is achieved following phosphorylation, ubiquitination and degradation of the I κ B proteins allowing the translocation of NF κ B homodimers into the nucleus where they can bind to regulatory components of target genes (Roff *et al.*, 1996; Sen *et al.*, 1986). For full activation, NF κ B must be further phosphorylated, and a number of different kinases have been implicated including the mitogen activated protein kinases (MAPKs) and protein kinase C (PKC) (Wajant *et al.*, 2003). Phosphorylation of I κ B is performed by the I κ B kinase (IKK) complex, which is composed of two catalytic sub-units, IKK α and IKK β (Mercurio *et al.*, 1997), as well as a regulatory sub-unit, NF κ B essential modulator (NEMO) (Yamaoka *et al.*, 1998) and a kinase specific chaperone (Chen *et al.*, 2002).

Previous work has demonstrated the presence of a DNA fragment upstream of the transcriptional start site of the TSLP gene containing a cis element required for transcriptional induction by IL-1 β . This sequence contains a consensus cognate element for NF κ B, and mutations within this element result in a reduction in IL-1 β induced upregulation of TSLP expression in a human lung epithelial cell line (Lee *et al.*, 2007a). EMSA studies further demonstrated IL-1 β -stimulation of these cells results in binding of the p65/p50 heterodimer of NF κ B to the human TSLP gene

promoter. In addition, expression of a dominant negative mutant of I κ B in the same epithelial cell line also inhibits IL-1 β mediated TSLP expression (Lee *et al.*, 2007a).

1.4.3.4. *Role of cooperative signaling in regulating TSLP expression*

The induction of TSLP at the protein level in NHBEs by IL-4 and IL-13 requires the presence of TNF- α , suggesting cooperative signalling between STAT6 and TNF- α related pathways (Kato *et al.*, 2007). Stimulation of TSLP expression evoked by rhinoviral dsDNA and RSV proteins via TLRs is also synergistically enhanced by IL-4, suggesting cooperative signalling between JAK/STAT and NF κ B pathways. This synergy between T-2 cytokines and TNF- α in inducing TSLP expression has also been demonstrated in human keratinocytes (Bogiatzi *et al.*, 2007). In support of the notion that STAT6 may be an important transcription factor in mediating TSLP expression is the observation that siRNA targeted against STAT6 attenuates the enhancement of dsRNA induced TSLP expression by IL-4 in NHBEs (Kato *et al.*, 2007).

A number of cellular targets of TSLP have been identified, including DCs, lymphocytes and granulocytes, as well as the more recent identification of structural cells that respond to TSLP. The relevance of these cells to the physiological and pathological roles of TSLP will now be discussed.

1.4.4. *The physiological role of TSLP*

Since TSLP was originally identified in conditioned media from a thymic stromal cell line, early studies focused on its potential role in lymphocyte development. However, *Tslpr*^{-/-} mice display normal T and B cell development, although mice deficient in both the TSLPR chain and the IL-2 receptor chain (*Tslpr*^{-/-} *Il2rg*^{-/-}) mice do show a more severe phenotype than *Il2rg*^{-/-} mice, suggesting a non-redundant influence of TSLP on lymphocyte development in mice (Al-Shami *et al.*, 2004; Carpino *et al.*, 2004). However, there exists evidence that, in humans at least, TSLP may play a role in the thymic selection of regulatory T-cells (Tregs) and in peripheral homeostasis of T cells.

TSLP is expressed by epithelial cells of Hassall's corpuscles (HCs) within the thymic medulla where it appears to activate a sub population of DCs (Watanabe *et al.*, 2005). It has been postulated that these DCs serve to positively select high-affinity self-reactive Tregs in the thymic medulla. Ordinarily, when high-affinity self-reactive T cells receive strong TCR signalling from epithelial cells within the thymic medulla,

they die by negative selection. In contrast, it is hypothesised that if these cells receive co-stimulatory signals, such as CD86, they will be converted into Tregs by secondary positive selection. DCs activated by TSLP express CD86 and *in vitro*, TSLP-activated DCs (TSLP-DCs) induce proliferation of CD4⁺CD8⁻CD25⁻ cells, of which 50% subsequently differentiate into CD4⁺CD8⁻CD25⁺FoxP3⁺ Tregs (Watanabe *et al.*, 2005). This hypothesis is also consistent with the *in vivo* localisation of Tregs within the thymic medulla in close proximity to activated DCs. In this regard, TSLP represents the only known activator DC maturation in the thymic medulla. However, further work is needed to clarify this postulated role.

TSLP-DCs are also capable of inducing a strong proliferation of naïve CD4⁺ cells (Watanabe *et al.*, 2004) and are found in close association with TSLP-expressing tonsillar epithelial cells. A further postulated physiological role for TSLP is in the peripheral homeostasis of T cells by peripheral mucosa lymphoid tissue (Watanabe *et al.*, 2004). The expansion of autologous naïve CD4 T cells by TSLP-DCs displays features of homeostatic expansion mediated by self-pMHC complexes, including dependence on MHC II and co-stimulatory molecules, but not on IL-7. Moreover, this expansion is a polyclonal expansion and importantly, the expanded population has the potential to further differentiate into Th1 or Th2 cells (Watanabe *et al.*, 2004). Once again, however, further studies are required in this field, and the remainder of this discussion will now focus on the role of TSLP in disease.

Briefly, as alluded to earlier, co-culture experiments with TSLP-DCs and naïve syngeneic CD4⁺ cells suggest a role for TSLP in T cell homeostasis. However, in allogeneic culture systems, T cells ultimately display characteristic features of differentiated inflammatory Th2 cells (Soumelis *et al.*, 2002). Moreover, TSLP-DCs are capable of supporting the maintenance and further polarisation of Th2 memory cells (Wang *et al.*, 2006), further supporting a role for TSLP in the development of T-2 inflammatory phenotype. These concepts will now be discussed in more detail, beginning with a summary of current understanding of Th2 cell differentiation.

1.4.5. Current concepts regarding the differentiation of CD4⁺ T lymphocytes

While T-cells, together with B-cells, are the key effector cells of the adaptive immune response, the initiation and subsequent modulation of their functions are achieved by DCs. These cells are professional antigen-presenting cells, and in this regard, their interaction with naïve T-cells is critical to activation of the latter, as T-cells cannot recognize antigen directly – rather, for activation and antigen-driven

proliferation, CD4⁺ T-cells require antigen to be processed and presented to their T-cell receptor (TCR) by MHC class II molecules on DCs. DCs have the capacity to polarise an immune response towards either a T-1 phenotype or a T-2 phenotype, depending on signals received at an immature stage from their microenvironment.

Naïve CD4⁺ cells have the potential to differentiate into Th1 or Th2 cells, in addition to Th17 and Tregs. These former 2 subsets are characterised by expression of their signature cytokines, IFN- γ and IL-4 respectively, following activation. The avidity of the TCR to the antigen/MHC complex presented on the DC, the nature of co-stimulatory molecules and the cytokine environment all influence the fate of a naïve CD4⁺ Th precursor cell, and the mechanisms involved in the differentiation of these cells into Th1 or Th2 lymphocytes will now be briefly discussed.

The initiation of Th1 differentiation begins with stimulation of naïve CD4 cells by antigen presenting cells, in particular activated DCs, via cognate TCRs. Critical to the development of Th1 cells is a cytokine milieu dominated by IL-12. Microbe-derived molecules can activate pattern recognition receptors on DCs, such as TLRs, leading to upregulation of IL-12 expression. In turn, IL-12 can act upon both natural killer (NK) cells and T cells to promote Th1 differentiation. IL-12 upregulates IFN- γ expression by NK cells (Yoshimoto *et al.*, 1998) which, via a STAT1 dependent mechanism, upregulates T-bet expression in naïve CD4 cells (Lighvani *et al.*, 2001). T-bet is a Th1 master transcription factor which induces IFN- γ production by CD4⁺ cells, as well as upregulating IL-12R β 2 expression (Mullen *et al.*, 2001). These T-bet^{hi} IL-12R β 2 expressing cells can therefore be selected for survival through the actions of IL-12, which via STAT4, further induces IFN- γ expression in this cell population. This integration of instructive and selective processes ultimately results in the clonal expansion of fully differentiated Th1 cells (Murphy *et al.*, 2000).

In terms of Th2 differentiation, IL-4 has long been recognised as a key cytokine involved in promoting the development of this CD4 subset *in vitro* (Le Gros *et al.*, 1990). More recently, the importance of IL-2 has also been highlighted in this differentiation program (Cote-Sierra *et al.*, 2004). As in Th1 differentiation, DC-derived signals are thought to influence the development of Th2 cells. However, activated DCs do not produce IL-4, and the primordial source of IL-4 *in vivo* remains unclear. It has been suggested that naïve T-cells themselves can produce limited amounts of IL-4 following interaction with low-strength signals (Grogan *et al.*, 2001; Yamane *et al.*, 2005). The strength of signal may be modulated by a number of means, including the presence or absence of co-stimulatory molecules such as

CD28; variations in the affinity of the peptide/MHC complex for the TCR and the duration of the DC-T cell interaction (Boyton *et al.*, 2002; Constant *et al.*, 1997; Iezzi *et al.*, 1999). Other possible cellular sources of IL-4 include NKT cells and basophils (Leite-de-Moraes *et al.*, 1997; Nouri-Aria *et al.*, 2001) though there remains insufficient evidence to invoke a role for these cells in the differentiation of Th2 cells.

IL-4 exerts its cellular effects via activation of STAT6, and a critical role for STAT6 in mediating the Th2-differentiating properties of IL-4 is illustrated by a severe block of Th2 differentiation in mice deficient in STAT6 (Takeda *et al.*, 1996). In addition to binding to the IL-4 promoter and 3' enhancer (Avni *et al.*, 2002), STAT6 promotes the expression of the Th2 master regulatory transcription factor, GATA-3 (Ouyang *et al.*, 2000). GATA-3 is a Th2-cell specific transcription factor which was identified as regulating the expression of a variety of Th2 cytokines, including IL-4, IL-5 and IL-13 (Zheng *et al.*, 1997). Its critical role in Th2 cell differentiation is demonstrated by observations that treatment with antisense oligonucleotide against GATA-3 represses Th2 development *in vivo* (Finotto *et al.*, 2001) and deletion of GATA-3 *in vivo* eliminates Th2 responses in mice (Zhu *et al.*, 2004). Importantly, GATA-3 has been reported to induce its own expression once its levels have reached a threshold level (Ouyang *et al.*, 2000). This feedback of transcriptional activation stabilizes GATA-3 levels within the cell. It has been postulated that non-IL-4/STAT6 mechanisms may serve to induce GATA-3 expression beyond this threshold. Thus, differentiating Th2 cells, following TCR engagement and activation of co-stimulatory signalling pathways, could themselves represent the initial source of IL-4 for amplification of this process. Such redundant signalling pathways include the CD28 and inducible T-cell co-stimulator (ICOS) pathways (Greenwald *et al.*, 2002). The mechanisms underlying GATA-3 regulation of Th2 gene expression is an expanding field and beyond the scope of this thesis. Briefly, however, GATA-3 binds directly to critical elements in the *Il5* and *Il13* promoters to upregulate their expression (Kishikawa *et al.*, 2001; Schwenger *et al.*, 2001). With respect to modulating *Il4* gene expression, GATA-3 creates heritable epigenetic patterns followed by the appearance of Th2-exclusive DNase hypersensitivity sites in the *Il4* locus (V/VA) and *Il4* / *Il13* intergenic region (conserved non-coding sequence (CNS)-1) (Zhu *et al.*, 2008). In addition, GATA-3 induces changes in the chromatin structure of the *Il4* gene (Lee *et al.*, 2000; Ouyang *et al.*, 2000).

These observations lend credence to the hypothesis that this master Th2 transcription factor mediates *Il4* gene expression, not by directly binding to promoter

regions, but rather, by chromatin remodelling and subsequent epigenetic modulation. De-repression of the *Il4* locus, renders it amenable to direct and immediate transcriptional control by non-Th2 specific transcription factors such as NFAT (Murphy *et al.*, 2002). The mechanisms described above do not, however, necessarily explain the clonal expansion of Th2 cells. GATA-3 mediates Th2 cytokine expression rather than cell growth, but the selective component to Th2 cell development may be explained by studies demonstrating that STAT6 induces expression of the zinc finger protein, GFI1, which in turn mediates clonal expansion (Zhu *et al.*, 2002). Furthermore, GFI1 only drives the proliferation of GATA-3hi cells, though the interaction between GATA-3 and GFI1 remains unclear (Zhu *et al.*, 2002). Despite the indispensability of GATA-3 in the development of Th2 responses *in vivo*, GATA-3 expression alone is not sufficient to induce IL-4 expression. As alluded to earlier, IL-2 is also critical to Th2 cell differentiation, and IL-2 mediated activation of STAT5a/STAT5b plays a critical role in inducing/maintaining accessibility at DNase hypersensitive sites of the *Il4* locus (Zhu *et al.*, 2003). Thus, it is likely that a tightly regulated collaboration between STAT5 and GATA-3 accounts for full Th2 differentiation *in vitro*. Finally, cross-regulation by cytokines and transcription factors reflects a further level of complexity to the differentiation of Th1/2 cells. For instance, GATA-3 downregulates STAT4 (Usui *et al.*, 2003), whereas T-bet can suppress GATA-3 expression (Usui *et al.*, 2006).

In summary, Th1 responses seem to be driven primarily by active signals derived from activated innate immune sources, such as DCs. However, the mechanisms underlying DC-mediated Th2 cell differentiation is less well delineated. The primordial source of IL-4, critical to an instructive and selective program of Th2 cell expansion, remains unknown, though it has been suggested that activated DCs that produce low amounts of IL-12 promote Th2 differentiation (Georas *et al.*, 2005). Environmental signals that lead to the maturation and activation of IL-12_{lo} DCs include low-dose endotoxin and TLR2 ligands (Georas *et al.*, 2005).

In an effort to delineate the signalling pathways upstream of significant IL-4 production by differentiating T cells, recent work has identified TSLP as a critical regulator of T-2 cytokine associated inflammatory diseases. TSLP is believed to be capable of instructing a programme of Th2 differentiation and proliferation via its unique actions on dendritic cells such that their subsequent interaction with naïve T-cells results in Th2 differentiation, independent of IL-4. These effects will now be discussed in more detail.

1.4.6. The role of TSLP in activating dendritic cells into a T-2 polarising phenotype

Activation of DCs is a critical step in engagement of the adaptive immune system to tissue injury or stress. Such activation may occur in an antigen-driven manner or, as has been described, in a non-antigenic manner, by danger signals reflecting tissue injury (Matzinger, 1994). Human myeloid DCs (mDCs) express the TSLP receptor complex (Soumelis *et al.*, 2002). TSLP strongly upregulates the expression of MHCII, CD80, CD83 and CD86 on human mDCs. In this regard, it is no different to other activators of DCs, such as CD40L, LPS, poly I:C and TLR ligands. Unlike these stimuli, however, TSLP does not stimulate mDCs to produce the Th1-polarizing cytokine, IL-12 (Ito *et al.*, 2005; Soumelis *et al.*, 2002). The molecular mechanisms underlying TSLP's ability to promote DC maturation without inducing IL-12 production remains unknown though the signalling pathways are likely to be independent of MyD88 and NF κ B transcription factors, both of which are required for the response to Th-1-promoting stimuli (Liu *et al.*, 2007b).

In allogeneic co-culture systems, TSLP-DCs induce the differentiation of naïve CD4⁺ T cells into inflammatory Th2 cells. In addition to the classical Th2 cytokines, IL-4, IL-5 and IL-13, these T cells secrete significant amounts of TNF- α . In contrast, they secrete little IFN- γ or IL-10, an anti-inflammatory cytokine (Soumelis *et al.*, 2002). Although not considered a Th2 cytokine, expression of TNF- α is often increased in inflammatory conditions associated with increased expression of T-2 cytokines (Moffatt *et al.*, 1997).

Recent studies have afforded some insight into the molecular mechanisms by which TSLP-DCs induce naïve CD4⁺ T cells to differentiate into inflammatory Th2 cells. TSLP is the only known activator of DCs to upregulate expression of OX40L, a member of the TNF superfamily (Ito *et al.*, 2005). Neutralisation of OX40L with an anti-OX40L antibody inhibits the production of T-2 cytokines and TNF- α by T-cells. Moreover, treatment of naïve CD4⁺ cells with human recombinant OX40L induces the expression of T-2 cytokines by naïve T cells, confirming that OX40L is the downstream molecule by which TSLP-DCs induce differentiation of inflammatory Th2 cells (Ito *et al.*, 2005). The signalling pathways downstream of OX40 ligation on naïve T cells remains unclear, though one study has demonstrated that OX40 signalling directly induces Th2 lineage commitment by activating nuclear factor of activated T cell (NFAT) c1 (So *et al.*, 2006). This triggers IL-4 production, which as

described in **Section 1.4.5.**, can then induce Th2 differentiation in a GATA-3 dependent manner (So *et al.*, 2006).

The ability of OX40L to induce differentiation of Th2 cells depends upon the absence of IL-12. In the presence of IL-12, OX40L loses this capability and instead, naïve T cells are directed towards Th1 differentiation (Ito *et al.*, 2005). Thus, the creation of a Th2-permissive microenvironment, through the absence of IL-12, represents a key characteristic of TSLP activation of DCs. These findings suggest that Th2 differentiation requires a negative signal (the absence of IL-12) and a positive signal (OX40L). Moreover, the observation that IL-12 is dominant over OX40L is compatible with the hygiene theory which postulates that microbial infections that trigger Th1 immune responses decrease the frequency of Th2-driven atopic conditions.

Although anti-OX40L antibody reduces the frequency with which naïve T cells differentiate into Th2 cells, this effect is not completely blocked. However, a complete switch to a Th1 phenotype is achieved when an anti-IL4 antibody is added. As TSLP-DCs do not produce IL-4, these findings suggest that while OX40L represents the initial Th2 polarising trigger, IL-4 functions as a key autocrine stabiliser and enhancer of *developing* Th2 cells.

TSLP also upregulates expression of CCL17 and macrophage derived chemokine (MDC) / CCL22 by immature mDCs (Soumelis *et al.*, 2002). These two chemokines are important in attracting Th2 cells [reviewed in (Homey *et al.*, 1999)], and may serve to amplify the Th2 cellular response at the site of initial activation of the DC. In addition, TSLP-DCs can induce a robust expansion of human Th2 memory cells, which maintain their central memory phenotype and capacity for effector cytokine function (Wang *et al.*, 2006), further supporting the notion that TSLP represents a critical regulator of pathological Th2 inflammatory responses.

1.4.7. Direct effects of TSLP on T cells

The manner by which TSLP can polarise the immune response by influencing the interactions between activated dendritic cells and naïve T cells has been described in **Section 1.4.6.** However, recent studies have also demonstrated that TSLP exerts direct effects on T cells *in vitro*. TSLP, in conjunction with TCR activation, is capable of driving Th2 differentiation of murine naïve CD4⁺ cells directly, in the absence of DCs and exogenous IL-4 (Omori *et al.*, 2007). These effects appear to be mediated by IL-4 acting downstream of TSLP and are compatible with previous work

demonstrating that TSLP can induce proliferation of CD4⁺ T cells (Al-Shami *et al.*, 2004). These findings have also been translated to human T cells, which are capable of proliferating in response to TSLP (Rochman *et al.*, 2007).

Aside from the aforementioned effects on naïve CD4⁺ cells, TSLP also exerts effects on Th2 effector cells. Recent studies have demonstrated that TSLP induces proliferation of murine effector Th2 cells and, together with TCR activation, is able to induce expression of IL-4 by effector Th2 cells (Kitajima *et al.*, 2011).

1.4.8. The role of TSLP in T-2 mediated inflammation: relevance to lung disease

Much of the work discussed in the preceding sections is supported by evidence from animal models of T-2 cytokine dominated diseases, and in certain instances, human disease relevance has been established, particularly in the context of allergic airways disease.

Pulmonary TSLP expression is increased in an ovalbumin (OVA)-induced murine model of allergic airways disease (Zhou *et al.*, 2005). Furthermore, in mice selectively expressing TSLP in alveolar epithelial cells, spontaneous T-2 biased airway inflammation is observed, with a predominantly eosinophilic alveolar infiltrate, accompanied by airway remodelling characterised by epithelial cell hyperplasia, subepithelial fibrosis and mucus metaplasia (Zhou *et al.*, 2005). In addition, TSLPR knockout mice, sensitized and challenged with OVA, demonstrate an attenuated airway inflammatory response (Zhou *et al.*, 2005). In these mice, reconstitution with TSLPR-sufficient T cells restores inflammatory disease, suggesting that TSLP is both necessary and sufficient for the development of a T-2 cytokine dominated inflammatory phenotype (Al-Shami *et al.*, 2005). In support of the notion that TSLP plays a crucial role in directing Th2 dominated inflammation is the observation that blockade of TSLP signalling in an OVA model of asthma, by the administration of anti-TSLPR antibodies (Shi *et al.*, 2008) or anti-TSLP neutralizing antibody (Li *et al.*, 2010), attenuated OVA-challenge induced airway inflammation – this attenuation was associated with reduced production of T-2 cytokines.

As mentioned above, in humans, atopic asthma represents a classical T-2 inflammatory condition. TSLP expression is significantly increased in the airways of asthmatic patients compared with normal control patients, and this increase correlates with the expression of T-2 chemokines and disease severity (Ying *et al.*, 2005).

However, the potential role of TSLP in promoting a cytokine milieu dominated by T-2 cytokines in non-allergen driven lung disease remains unknown. In light of the evidence presented above that TSLP is a key activator of DCs into a T-2 polarizing phenotype, the role of this cell type in IPF will now be discussed in more detail.

1.5 The role of dendritic cells in IPF

Immature DCs are widely distributed *in vivo* to optimize antigen capture, and given the extremely large alveolar space that is at the interface between the host and the environment, they have a critical role in orchestrating the adaptive immune response to antigens presented to the lung. DCs are widely distributed throughout the normal lung, continuously sampling exogenous antigen, before trafficking to local lymph nodes to present them to naïve T-cells. An increase in the lung DC population is seen in a number of respiratory diseases, including COPD, lung cancer and lung transplant rejection (Vermaelen *et al.*, 2005), though they have been most extensively studied in atopic asthma, where a clear pathogenetic role has been identified (van Rijt *et al.*, 2005). As mentioned earlier, DCs have the capacity to polarise the immune response towards a T-1 or T-2 phenotype, depending upon signals received at an immature stage from their microenvironment, and while these signals had initially been thought to be solely antigen-derived, recent work suggests that DCs may also be activated by non-antigenic danger signals (Matzinger, 1994), via purinergic receptors and TLRs [reviewed in (Skoberne *et al.*, 2004)]. Such signals may be endogenous, and related to cellular degradation eg. heat shock proteins (HSPs) (Srivastava, 2002), ATP (Wilkin *et al.*, 2001) and uric acid (Shi *et al.*, 2003); endogenous and related to disruption of normal tissues architecture e.g. heparin-sulphate proteoglycans (Johnson *et al.*, 2002); or exogenous pathogen-derived TLR ligands, such as viral dsRNA and LPS (Kaisho *et al.*, 2003).

As alluded to in **Section 1.3.2.2.**, the importance of inflammasome activation by danger signals released following lung injury in promoting lung fibrosis has recently been demonstrated (Gasse *et al.*, 2009). In these studies, NALP3 inflammasome activation, and subsequent maturation of IL-1 β , following lung injury was demonstrated to be dependent upon the release of uric acid from injured cells. Strategies designed to lower uric acid levels, such as allopurinol or uricase administration, prevented activation of the inflammasome and attenuated bleomycin-induced lung fibrosis (Gasse *et al.*, 2009). The potential importance of this pathway in human disease is supported by the observation that uric acid levels

are elevated in IPF lung compared with non-fibrotic control lung (Markart *et al.*, 2009). Moreover, uric acid has been demonstrated to be an important endogenous danger signal released from injured cells that activates immature dendritic cells and subsequent downstream immune responses (Shi *et al.*, 2003). Another danger signal common to activation of the NALP-3 inflammasome (Aymeric *et al.*, 2010), fibrogenesis (Riteau *et al.*, 2010) and dendritic cell activation (Wilkin *et al.*, 2001) is extracellular ATP (eATP). Levels of eATP are elevated in broncho-alveolar lavage fluid (BALF) specimens from IPF patients compared to non-fibrotic control lung and eATP promotes maturation of IL-1 β and evolution of inflammation to fibrosis in an animal model of lung injury - the source of eATP in this model was postulated to be injured epithelial cells (Riteau *et al.*, 2010). Collectively, these data support the notion that danger signals released from injured epithelium, not only activate the innate immune system but are additionally perceived by dendritic cells, thus engaging the adaptive immune system.

In light of the importance attached to chronic epithelial cell injury in the pathogenesis of IPF, this thesis will address the overarching hypothesis that DCs, activated by these danger signals, may promote a local pro-fibrotic immune response at sites of tissue damage. The IPF lung has been reported to be infiltrated by mature/activated dendritic cells (Marchal-Somme *et al.*, 2006), located adjacent to T-cell aggregates in a peri-vascular distribution. Moreover, these activated DCs were observed to be located in close proximity to T-cells expressing CD40L, a terminal maturation marker to DCs (Quezada *et al.*, 2004), suggesting that such maturation might occur *in situ*. Recent work has also demonstrated that the IPF lung is also heavily infiltrated by immature DCs, expressing Specific Intercellular adhesion molecule-3-Grabbing Non-Integrin (DC-SIGNs), in a peri-bronchovascular distribution, in close proximity to endothelial cells expressing ICAM-2, critical for DC-SIGN mediated DC trafficking (Geijtenbeek *et al.*, 2000). In addition, DC-SIGN expressing DCs were observed at the periphery of T-cell aggregates expressing ICAM-3 (Marchal-Somme *et al.*, 2007a), another important SIGN ligand (van Kooyk *et al.*, 2002). Furthermore, hyperplastic epithelial cells and fibroblasts in IPF lung displayed strong immunoreactivity for chemokines known to be important in mediating DC recruitment, including CCL19, CCL22 and CXCL12 (Caux *et al.*, 2000). These latter findings support the notion of a “stromal address code” (Buckley *et al.*, 2001) the aberrant expression of which leads to the accumulation of DCs and lymphocytes within inflamed organs. This phenomenon is well-recognised in other human auto-immune diseases such as rheumatoid arthritis (Parsonage *et al.*, 2005).

Traditionally, the interaction between DCs and naïve T-cells has been thought to occur in local lymph nodes, to where the recently activated and maturing DCs traffic; certainly, mature DCs rapidly accumulate in lymph nodes under inflammatory conditions (Cook *et al.*, 2007). However, the accumulation of such cells in the lung following BLM challenge suggests that they have the potential to interact with T-cells in tertiary lymphoid structures, consistent with previous reports that migration of DCs to lymph nodes is not a prerequisite for lung T-cell activation (Constant *et al.*, 2002; Wakim *et al.*, 2008).

As described above, mature DCs play a critical role in the activation of T-lymphocytes, and their subsequent polarisation into Type 1 or Type 2 T-cells. The following section will address the potential role of T-cells in the pathogenesis of lung fibrosis.

1.6. The role of the adaptive immune response in lung fibrosis

T-lymphocytes are a key cellular component of the inflammatory response and, in particular, of the adaptive immune response. While their role in orchestrating immune responses linked to fibroproliferation in other organs is well established (Marrack *et al.*, 2001), evidence regarding their involvement in lung fibrosis is less clear. The basis for their immune function is selective clonal expansion following specific antigen exposure. Such oligoclonal expansion is seen in fibroproliferative diseases of the lung, such as berylliosis, where a clear role for T-cells has been established (Fontenot *et al.*, 1999), and such findings have recently been observed in IPF patients (Feghali-Bostwick *et al.*, 2007; Shimizudani *et al.*, 2002). Evidence regarding their potential role in the pathogenesis of IPF will now be discussed.

1.6.1. T-cells in animal models of pulmonary fibrosis

Data from animal models provide conflicting evidence as to the potential importance of T-cells of lung fibrosis. Lung T-cell accumulation has been reported to be increased in mice following bleomycin challenge from day 6 onwards compared to saline-treated controls (Izbicki *et al.*, 2002; Zhu *et al.*, 1996). Lung collagen accumulation in a bleomycin model of fibrosis is attenuated in athymic nude mice compared to wild type controls (Schrier *et al.*, 1983). A similar attenuation is seen in *rag*^{-/-} mice (Oikonomou *et al.*, 2006), which are completely devoid of both T and B cells, and lymphocyte recruitment is associated with the development of bleomycin-induced fibrosis, suggesting a role for the cellular immune system in this model

(Oikonomou *et al.*, 2006). Furthermore, mice that lack CD28, a critical co-stimulatory molecule required for full T-cell activation, are protected from BLM-induced fibrosis; susceptibility is restored following the adoptive transfer of CD28 expressing T cells (Okazaki *et al.*, 2001), strongly suggesting that T-cell activation is required for the development of fibrosis following lung injury.

Systemic depletion of T-cells in mice can be achieved by the administration of anti-CD3, anti-CD4 and/or anti-CD8 antibody, and in these instances, mice are protected from BLM-induced fibrosis (Huaux *et al.*, 2003b; Piguet *et al.*, 1989; Sharma *et al.*, 1996). However, these findings are not universal (Janick-Buckner *et al.*, 1989b) and a number of groups have failed to demonstrate any protection from bleomycin-induced fibrosis in mice lacking T-cells (Helene *et al.*, 1999; Szapiel *et al.*, 1979) – one possible explanation for these discrepancies may be that severe combined immunodeficiency (SCID) mice, used in a number of these studies, are somewhat leaky, with residual circulating lymphocytes. It must also be noted that the dose of bleomycin, as well as the route of its administration varied from study to study. It is also plausible that variability of results from studies using knockout mice is due to the development of compensatory immune and repair mechanisms in an environment-dependent manner. Furthermore, the conflicting results observed with studies employing anti-CD3, anti-CD4 and/or anti-CD8 antibody administration may be due to variability in efficiency in terms of T-cell depletion. A limitation of the genetic models and those employing systemic depletion of T-cells, as described above, is that T-cells accumulate as part of a complex inflammatory response, thus confounding any specific role they may play in fibrogenesis. This issue may be circumvented by the selective attraction of T-cells to healthy lung. Transgenic mice overexpressing CCL18, a highly specific T-cell chemoattractant develop lung fibrosis in close association with T-cell infiltrates and importantly this effect is attenuated by the systemic depletion of T-cells (Luzina *et al.*, 2006).

Although the precise profile of T-cells that have been reported to accumulate in the lung following experimentally-induced lung injury remains unclear, recent work suggests that, in the bleomycin, they are predominantly of a memory phenotype, (Bantsimba-Malanda *et al.*, 2010), consistent with the hypothesis that lymphocytes in the lung are memory cells whose population is maintained by continuous recruitment (Ely *et al.*, 2006). Such memory T cells have the potential to be reactivated locally by DCs under inflammatory conditions (Heath *et al.*, 2009), and importantly retain the capacity for effector cytokine generation (Harris *et al.*, 2002).

Furthermore, these T cells also express CD40L, a marker of recently activated T-cells (Quezada *et al.*, 2004), but not Ki67, a marker of proliferation, suggesting activation prior to recruitment to the aggregates (Bantsimba-Malanda *et al.*, 2010).

1.6.2. T-cells in IPF

Despite controversy surrounding the role of inflammation in IPF, there are numerous reports to suggest a role for T-cells in the pathogenesis of IPF. The presence of T cells is consistently observed in lung tissue and BALF of IPF patients (Daniil *et al.*, 2005; Papiris *et al.*, 2005; Parra *et al.*, 2007) and their presence has been reported to correlate with worse lung function and poor survival (Daniil *et al.*, 2005). Accumulation of T-cells appears to correlate with the degree of fibrosis, with relatively few T cells observed in areas of normal lung architecture (Parra *et al.*, 2007). Their distribution is variable and ranges from a diffuse infiltration throughout alveolar spaces and interstitium to focal perivascular aggregates (Luzina *et al.*, 2008).

Recent work has demonstrated that peripheral CD4+ T-cells from IPF patients are more frequently activated than similar cells from healthy volunteers, with increased surface expression of MHC II and CD40L (Feghali-Bostwick *et al.*, 2007). Interestingly, analysis of the TCR repertoires of these cells suggests that observed abnormal TCR-variable beta (TCRBV) expansions were due to clonal proliferation, suggesting oligoclonal expansions of these activated CD4 T-cells (Feghali-Bostwick *et al.*, 2007). Similar analyses of BALF T-cells from IPF patients (Shimizudani *et al.*, 2002) support the notion that IPF is associated with an oligoclonal expansion of T-lymphocytes, suggesting antigenic stimulation of these cells (Becker *et al.*, 2000).

The presence of lymphoid follicles, resembling germinal centres, in IPF lung has been noted for some time (Campbell *et al.*, 1985; Wallace *et al.*, 1996). Germinal centres are essential for lymphoid neogenesis, and consist of proliferating B cells and T-cells (Weyand *et al.*, 2001) When occurring ectopically, they are termed tertiary lymphoid follicles. These follicles are found in a number of chronic inflammatory human diseases (Hjelmstrom, 2001), but only recently have their structure and function been evaluated in IPF. As alluded to earlier, recent work has demonstrated significant peri-vascular lymphocytic infiltration in IPF lung in which the lymphocytes were organized in follicular-type structures (Marchal-Somme *et al.*, 2006). Within these structures, T-lymphocytes were predominantly CD4+, expressing a CD45RO+ memory phenotype. Importantly, the vast majority were

also CD40L⁺. As described above, expression of this ligand is upregulated soon after T-cell activation and serves as a key maturation signal to dendritic cells (Quezada *et al.*, 2004). This preponderance of CD40L⁺ memory T-cells, in both animal models of fibrosis, as well as in IPF lung, suggests that the T-lymphocytes infiltrating IPF lung are activated and antigen-experienced, hinting at a possible role for the adaptive immune response to the fibrogenesis, and in this regard, the role of auto-immunity in IPF will now be discussed.

1.6.3. Auto-immunity in IPF

In support of the notion that autoimmunity might be involved in the pathogenesis of IPF are reports of circulating immune complexes and autoantibodies against a variety of self-antigens in IPF patients (Chapman *et al.*, 1984; Dobashi *et al.*, 2000; Feghali-Bostwick *et al.*, 2007; Yang *et al.*, 2002). It has been postulated that the AEC represents a potential target of an autoimmune process in IPF, as a number of these circulating antibodies are directed against epithelial cells, including those very recently identified targeting periplakin (PPL) (Taille *et al.*, 2010), a member of the plakin protein family which localises to desmosomes and intermediate filaments. The interaction between PPL and intermediate filaments is required for closure of experimental wounds in epithelial monolayers (Long *et al.*, 2006) - the observation that anti-PPL antibodies inhibited epithelial cell repair *in vitro* supports the hypothesis that impaired epithelial reconstitution following injury might be a key step in the pathogenesis of lung fibrosis (Taille *et al.*, 2010).

Importantly, *in vitro* co-culture experiments have demonstrated that IPF lung extract stimulates proliferation of autologous CD4⁺ T-cells in contrast to preparations from healthy controls, strongly suggestive of an underlying autoimmune phenomenon in IPF patients (Feghali-Bostwick *et al.*, 2007). Two recent studies provide further support for a role of the adaptive immune response in the pathogenesis of IPF. First, marked downregulation of CD28 expression has been demonstrated on circulating CD4⁺ T-lymphocytes from IPF patients, in contrast to T-cells derived from healthy individuals, almost all of which express CD28 (Gilani *et al.*, 2010). As alluded to **Section 1.6.1.**, CD28 serves as a key co-stimulatory molecule to ensure complete activation of naïve CD4⁺ T-cells. However, following repeated cycles of antigen-driven proliferation, human CD4⁺ T-cells develop a number of phenotypic and functional characteristics, including loss of CD28 expression (Hirokawa *et al.*, 2001; Studer *et al.*, 2008); these activated CD4⁺ T-cells, or memory T-cells, do not require CD28 for further activation and effector cytokine function (Fontenot *et al.*,

2003). The loss of CD28 expression by circulating CD4⁺ T-cells in IPF is strongly suggestive of an underlying chronic adaptive immune response, suggesting the presence of an offending antigen as an initiating injurious stimulus, in the pathogenesis of IPF. Indeed, the downregulation of CD28 expression appears to be associated with poor outcomes in these patients (Gilani *et al.*, 2010). Second, circulating and BALF FoxP3⁺ Tregs, which are essential for the control of immunological tolerance and the dampening of the adaptive immune response against antigen, are reduced in number in IPF patients compared with non-IPF controls (Kotsianidis *et al.*, 2009). Importantly, the suppression of experimentally-induced T-2 immune responses *in vitro* by Tregs derived from IPF BALF is significantly impaired. Moreover, this degree of impairment correlates with lung function parameters (Kotsianidis *et al.*, 2009). Such quantitative and qualitative defects in Treg populations are well described in other human autoimmune diseases (Ehrenstein *et al.*, 2004), pointing to a similar pathogenetic process in IPF.

1.7. Mechanism of action of T-cells in pulmonary fibrosis

While a defect in Treg number and function suggests a deficiency in immune tolerance that may contribute to the pathogenesis of IPF, the mechanism of action of non-Treg CD4⁺ T-cells in this condition remains unclear. Several studies have demonstrated that activated T-cells can directly induce collagen synthesis by fibroblasts (Cathcart *et al.*, 1987; Selman *et al.*, 1990; Yamamura *et al.*, 2001). One possible mechanism may involve the CD40 ligand (CD40L)-CD40 activation pathway. CD40L expression is higher on IPF CD4⁺ T-cells, compared to control cells (Feghali-Bostwick *et al.*, 2007), and interaction between CD40L and its cognate receptor, CD40, results in increased collagen synthesis by CD40 expressing lung fibroblasts (Sempowski *et al.*, 1997). In addition to membrane-dependent interactions, T-cells may regulate fibrogenesis via the generation chemokine gradients promoting the recruitment of fibrocytes to the lung. For instance, activated CD4⁺ T-cells can elaborate CCL3 (Grob *et al.*, 2003), and CCL3^{-/-} mice, as well as mice lacking its receptor, CCR5, are protected from BLM-induced fibrosis, with an associated attenuation in lung fibrocyte recruitment (Ishida *et al.*, 2007). An expansion of a functional fibrocyte population may also be achieved through differentiation of monocytes, and the attenuation of experimentally-induced renal fibrosis observed with anti-CD4 antibody treatment is associated with a reduction in fibrocyte differentiation, suggesting that both fibrocyte recruitment and differentiation is critically dependent on CD4⁺ T cell activity (Niedermeier *et al.*, 2009). However, most attention regarding the role of T-cells in fibrosis have focused

on their expression of soluble T-2 cytokines. The pro-fibrotic potential of these cytokines will now be discussed in more detail.

1.7.1. The T-2 hypothesis of fibrosis

There is a growing body of evidence to suggest that the cytokine phenotype of the immune response to an inciting stimulus determines whether injury progresses to fibrosis or resolves. This hypothesis proposes that progressive fibrosis results from a maladaptive immune response to a chronic or persistent antigen or inciting agent(s). The fibroproliferative response to injury can therefore be considered a result of an inappropriate shift towards a more T-2 phenotype characterised by a relative increase in expression of T-2 cytokines, such as IL-13, over T-1 cytokines such as IFN- γ (Wynn *et al.*, 1995). This polarisation is independent of the potency of inflammation as potent inflammatory responses dominated by IFN- γ induced in animal models of schistosomiasis do not result in fibrosis (Hoffmann *et al.*, 2000). Rather, this hypothesis proposes that fibrogenesis is regulated by the nature of the cytokine phenotype of the immune response. Moreover, it has been argued that a switch to T-2 cytokine expression may be in the interests of the host organism when faced with persistent T-1 dominated responses to prevent excessive inflammation-induced damage and death (Wynn, 2004). In a murine model of schistosomiasis, IL-10/IL-4 knockout mice, which develop a highly polarised T-1 phenotype, demonstrate 100% mortality at week 9 following egg-laying, strongly linked to IFN- γ levels, in contrast to T-2 biased mice, which display only 30% mortality at the same time point (Hoffmann *et al.*, 2000). In this regard, the development of organ fibrosis may be an acceptable compromise to ensure survival, and thus be viewed as an adaptive wound healing mechanism.

1.7.2. The role of IL-13 in pulmonary fibrosis

Of all the T-2 cytokines, IL-13 has received most attention in the context of several fibrotic conditions [reviewed in (Wynn, 2004)]. IL-13 is predominantly secreted by Th2 cells, but other cellular sources include mast cells, basophils and eosinophils. More recently, human alveolar epithelial cells and macrophages have also been identified as significant sources of IL-13 (Allahverdian *et al.*, 2008; Hancock *et al.*, 1998).

The hallmark of lung fibrosis is the inappropriate deposition of excessive ECM proteins present in the interstitium, and the predominant such protein is collagen. This net excess reflects increased synthesis, though reduced turnover is also

contributory (McAnulty *et al.*, 1987). In addition to being mitogenic for human fibroblasts (Saito *et al.*, 2003), IL-13 induces the upregulation of total collagen production and upregulates collagen type I gene expression by this key effector cell of fibrosis (Oriente *et al.*, 2000). Furthermore, IL-13 can promote differentiation of fibroblasts into myofibroblasts (Saito *et al.*, 2003). IL-13 may also promote a non-degradative micro-environment within the pulmonary interstitium, by its ability to inhibit IL-1 β induced expression of matrix metalloproteinase (MMP)-1 by fibroblasts and by upregulating tissue inhibitor of metalloproteinase (TIMP)-1 expression (Oriente *et al.*, 2000). More recently, IL-13 has been identified as a mediator of epithelial cell apoptosis *in vitro* (Borowski *et al.*, 2008), suggesting a role for this T-2 cytokine in amplifying the dysregulated crosstalk between the epithelium and the mesenchyme.

In animal models, bleomycin-induced lung injury induces upregulation of IL-13 protein expression. The time of maximal upregulation appears to be between 12 and 28 days post challenge (Belperio *et al.*, 2002; Jakubzick *et al.*, 2003). Moreover, IL-13^{-/-} mice are protected from bleomycin-induced fibrosis at day 21 (Liu *et al.*, 2004b), whereas selective airway expression of IL-13 in mice promotes the development of sub-epithelial fibrosis at one month of age and of parenchymal fibrosis by three months (Lee *et al.*, 2001). Further support for a role for IL-13 in fibroproliferative lung disease in animal models, comes from studies targeting IL-13 or IL-13 responsive cells. Attenuation of bleomycin-induced fibrosis in mice is observed by passive neutralization of IL-13 with anti-IL-13 antibodies (Belperio *et al.*, 2002), and a similar observation is seen following the therapeutic administration of an IL-13 immunotoxin chimeric molecule (IL13-PE) comprised of human IL-13 and a mutated form of *Pseudomonas aeruginosa* exotoxin A (Jakubzick *et al.*, 2003).

As well as exerting direct effects on epithelial cells and fibroblasts, IL-13 may promote a fibrotic phenotype via a number of other mediators. IL-13 induction of TGF- β expression has been demonstrated in macrophages (Fichtner-Feigl *et al.*, 2006), and IL-13 also seems capable of activating latent TGF- β via a plasmin/serine and MMP-9 dependent manner (Lee *et al.*, 2001). Found in Inflammatory Zone (FIZZ) -1 is a protein that belongs to a novel family of cysteine rich secreted proteins termed resistin-like molecules (RELMS). FIZZ-1 is a potent inducer of fibroblast to myofibroblast differentiation *in vitro* (Liu *et al.*, 2004a), and its expression is significantly upregulated in epithelial cells following bleomycin-induced lung injury in

mice. However, this upregulation is completely abrogated in IL-13 knockout mice which are protected from bleomycin-induced fibrosis (Liu *et al.*, 2004b).

In humans, elevated levels of macrophage-derived IL-13 have been observed in BALF samples from IPF compared to control (Hancock *et al.*, 1998). Since then, elevated IL-13 mRNA and protein levels have been reported in lung biopsies of patients with IPF compared to non-fibrotic control biopsies (Jakubzick *et al.*, 2004b). This latter group also demonstrated a significant increase in IL-13 receptor expression in IPF lung compared to non-fibrotic control, localising in particular to fibrotic foci leading to the suggestion that this may facilitate increased responsiveness to IL-13 of the cellular components of such foci. Indeed, lung fibroblasts derived from IPF lung exhibit greater IL-13 receptor expression than non-fibrotic control cells and display greater susceptibility to IL-13PE-mediated cytotoxicity *in vitro* (Jakubzick *et al.*, 2004a). Collectively, these findings support the notion that IL-13 may play a significant role in the pathogenesis of IPF which is reflected by an on-going interest in targeting IL-13 as a novel therapeutic strategy in IPF and other fibrotic conditions (Datta *et al.*, 2011). IL-13PE has recently been granted orphan drug status in the USA, and an IL-13 neutralising antibody is currently being evaluated in IPF patients (Novartis, USA).

1.7.3. The role of IL-4 in fibroproliferative disease

Although IL-13 has received most attention of the T-2 cytokines in the pathogenesis of fibrotic conditions, several studies have also demonstrated the potential importance of the prototypic T-2 cytokine, IL-4, in such diseases. IL-4 exerts a wide range of effects on a number of different cell types. For example, it is a key cytokine involved in driving the differentiation of naïve Th cells into Th2 cells, and is crucial for eosinophil chemotaxis. IL-4 exerts a number of regulatory effects on human fibroblasts, including promoting chemotaxis, proliferation, collagen synthesis and differentiation into myofibroblasts (Postlethwaite *et al.*, 1992; Postlethwaite *et al.*, 1991; Sempowski *et al.*, 1994). IL-4 expression is upregulated in mice following bleomycin-induced lung injury (Gharaee-Kermani *et al.*, 2001), and human disease relevance is suggested by the observation of enhanced IL-4 immunoreactivity in IPF lung sections, compared to normal controls (Ando *et al.*, 1999; Wallace *et al.*, 1995). However, the role of IL-4 in the pathogenesis of lung fibrosis appears more complex than previously thought. For example, it appears to possess immunosuppressive and anti-inflammatory properties, and has been demonstrated to inhibit TNF- α synthesis by macrophages (Levings *et al.*, 1999). In accordance with this, IL-4 appears to perform a dual role following lung injury in murine models :

at early time points following bleomycin-induced injury, it is immunosuppressive and anti-inflammatory contributing to limited T-cell recruitment and reduced TNF- α generation, but thereafter appears to be pro-fibrotic (Huaux *et al.*, 2003a).

1.7.4. The role of T-2 chemokines in fibroproliferative disease

It is increasingly recognised that chemokines can cooperate with pro-fibrotic T-2 cytokines in the development of fibrosis, via the recruitment of lymphocytes, macrophages and other effector cells to sites of tissue damage. Together with their receptors, chemokines are an essential component of T-1 and T-2 mediated immune responses, and their importance in the polarisation and subsequent amplification of a particular immune response is reflected by the differential expression of chemokine receptors on Th1 and Th2 cells: CXCR3 and CCR5 are predominantly expressed by Th1 cells whereas Th2 cells are characterised by increased CCR4 and CCR8 expression (Sallusto *et al.*, 1998). It is likely that numerous chemokine signalling pathways are involved in fibrogenesis, but the CC-chemokine family has been most extensively studied. Pathogenic roles for CCL6 and CCL17 have been proposed on the basis of animal studies demonstrating attenuated fibrotic responses to bleomycin following passive neutralization of these chemokines (Belperio *et al.*, 2002; Belperio *et al.*, 2004). The attenuation in bleomycin-induced fibrosis observed with anti-CCL6 antibody administration is associated with a reduction in macrophage infiltration (Belperio *et al.*, 2002). The importance of macrophages to the development of pulmonary fibrosis is well documented and beyond the scope of this thesis. Briefly, however, macrophages are felt to be a major cellular source of pro-fibrotic cytokines such as TGF- β and PDGF (Keane MP, 2000) as well as MMPs, important mediators of ECM remodelling (Riches, 2000). Indeed, macrophages appear to be the predominant cellular source of CCL6 in this model, downstream of IL-13 activity, suggesting an autocrine pathway to amplify the inflammatory response to lung injury. A similar association between reduced macrophage infiltration and fibrosis following bleomycin-induced lung injury has also been observed following neutralisation of CCL17 (Belperio *et al.*, 2004). Interestingly, this was also associated with a significant reduction in lymphocyte accumulation and indeed, expression of CCR4, the cognate CCL17 receptor, was increased in this model. Conversely, CXCR3^{-/-} knockout mice demonstrate increased mortality with progressive fibrosis in a bleomycin model of lung injury compared to wild type controls (Jiang *et al.*, 2004). Importantly, human disease relevance for the importance of CCR4 in the pathogenesis of IPF has recently been suggested by the observation of increased

CCR4 expression on BALF CD4⁺ T-cells in IPF lung compared to control, with a concomitant reduction in CXCR3 expression on the same T-cell population (Pignatti *et al.*, 2006).

Arguably the most intensively studied CC-chemokine in lung fibrosis, however, is CCL2. This chemokine is produced by a number of cell types, including epithelial cells and fibroblasts (Puneet *et al.*, 2005) and is a potent chemoattractant for T cells, immature dendritic cells and mononuclear cells (Rose *et al.*, 2003). Indeed, fibroblast-derived CCL2 has recently been demonstrated to play a critical role in dendritic cell trafficking in the lung following experimentally-induced airway inflammation and remodelling (Kitamura *et al.*, 2011). Lung CCL2 expression is increased in mice following bleomycin challenge (Zhang *et al.*, 1994a), and mice deficient in the unique CCL2 receptor, CCR2, are protected from BLM-induced lung fibrosis (Gharaee-Kermani *et al.*, 2003). Elevated CCL2 expression has been reported in IPF (Antoniades *et al.*, 1992; Mercer *et al.*, 2009) and lung fibroblasts derived from fibrotic lesions of IPF patients display a greater ability to express CCL2 compared to normal control fibroblasts (Standiford *et al.*, 1993).

As discussed in **Section 1.3.2.3.**, fibrocytes have been proposed to contribute to the myofibroblast population in IPF. Fibrocytes have been reported to accumulate in the lung following bleomycin-induced lung injury. In this model, the protection afforded from fibrosis in mice deficient in the receptor for CCL2, CCR2, is associated with reduced fibrocyte and monocyte recruitment (Moore *et al.*, 2005), highlighting the importance of CCR2 signalling axis in this regard. CCL2 may further contribute to excessive matrix deposition by stimulating fibroblast collagen production via TGF- β (Gharaee-Kermani *et al.*, 1996). CCL2 has also been reported to engage in a tripartite relationship with TGF- β and IL-13, with interplay between these three cytokines promoting a pro-fibrotic response (Murray *et al.*, 2008).

In addition, recent studies have suggested that CCL2 may amplify the dysregulated epithelial-mesenchymal crosstalk following lung injury via its ability to regulate apoptosis. As discussed in **Section 1.3.2.3.**, resistance of lung (myo)fibroblasts to apoptosis may contribute to the persistence of this key effector cell in lung fibrosis; CCL2 mediates such resistance *in vitro* in an IL-6 dependent manner (Liu *et al.*, 2007a).

Over the past 15 years, it has become increasingly apparent that chemokines may also contribute to regulation of the immune response (Luther *et al.*, 2001). T-cell

activation in the presence of CCL2 results in enhanced T-2 cytokine generation (Karpus *et al.*, 1997). Moreover, neutralization of fibroblast-derived CCL2 attenuates IL-4 expression by T-cells with a concomitant increase in expression of the prototypic T-1 cytokine, interferon (IFN)- γ (Hogaboam *et al.*, 1998). These findings, demonstrating the T-enhancing properties of CCL2, are supported by *in vivo* studies in which CCL2^{-/-} mice are unable to mount T-2 immune responses (Gu *et al.*, 2000).

Taken together, these findings suggest that CCL2 represents an important mediator in fibrotic lung disease by promoting a range of cellular responses.

1.8. The role of TNF- α in fibroproliferative disease

The work presented in this thesis focuses on TNF- α as a regulator of TSLP expression. An overview of TNF- α biology will therefore now be provided. TNF- α is a pluripotent cytokine that has traditionally been viewed as being pro-inflammatory. However, it may be more appropriately regarded as a master cytokine involved in a wide range of cellular processes including differentiation, proliferation and apoptosis. Its basic biology and relevance to fibroproliferative disease of the lung will now be discussed.

1.8.1. TNF- α : a brief overview of biology

In humans, TNF- α is initially synthesised as a 26kDa pro-protein which is integrated into the cell membrane (mTNF) existing as a stable homotrimer (Tang *et al.*, 1996). This cell associated form is biologically active and is thought to be involved in juxtacrine signalling following initiation of cell-cell contact (Kriegler *et al.*, 1988). In addition, a further biologically active form is created by the action of matrix metalloproteinases which cleave the extracellular domain releasing a mature 17kDa soluble protein (sTNF) which also exists in a homotrimeric form (Black *et al.*, 1997). The major enzyme involved in releasing sTNF from mTNF is TNF- α converting enzyme (TACE) (Moss *et al.*, 1997). The particular functions of mTNF and sTNF remain unclear, though recent evidence suggests in certain instances they may play distinct roles. For instance, sTNF knockout mice are protected from endotoxin-induced lethality (Josephs *et al.*, 2000) but are sensitive to the development of chronic inflammatory conditions such as rheumatoid arthritis (Alexopoulou *et al.*, 1997) and the relevance of this to fibroproliferative disease of the lung will be discussed in more detail in **Section 1.8.2**. To a certain degree this may reflect a preference for sTNF to signal via one of 2 structurally distinct TNF- α receptors, TNF receptor type 1 (TNFR1), over TNFR2 (Grell *et al.*, 1995), as TNFR1 knockout mice

are also resistant to endotoxin-induced lethality (Evans *et al.*, 1994). The reason for this remains unclear, though may reflect differing on-off kinetics of the two receptors – TNF- α binding to TNFRI is essentially irreversible, whereas binding to TNFRII is associated with rapid on-off kinetics (Grell *et al.*, 1998).

The distribution of these receptor sub-types varies between different tissues and cell types. TNFRI is constitutively expressed in most tissues whereas TNFRII expression is tightly regulated and is typically limited to cells of the immune system. The majority of cellular processes induced by TNF- α are mediated via TNFRI. The binding of TNF- α to TNFRI triggers a series of intracellular events which ultimately result in the activation of two major transcription factors, NF κ B and AP-1. As discussed in **Section 1.4.3.**, these transcription factors are responsible for the inducible expression of genes important for a wide range of cellular processes including cell growth and development as well as host immune and inflammatory responses.

As discussed in **Section 1.4.3.3.**, liberation of NF κ B from I κ B is achieved following the phosphorylation-induced degradation of I κ B, to which it is bound in the cytoplasm. This phosphorylation of I κ B is performed by the I κ B kinase (IKK) complex. The initial step in TNF-induced activation of the IKK complex is a conformational shift of the TNF-R1 complex, resulting in release of the inhibitory protein, silencer of death domain (SODD) (Jiang *et al.*, 1999). TNFRI possess an intra-cellular protein-protein interaction domain termed death-domain (DD). This DD can recruit other DD-containing proteins. Following SODD dissociation, the DD-containing adaptor protein, tumour necrosis factor receptor type 1-associated death domain protein (TRADD), is recruited to the death domain (DD) of TNFRI by homophilic interactions between these DDs (Hsu *et al.*, 1995). TRADD, in turn, serves as a scaffold for the binding of TNF receptor-associated factor (TRAF)-2, and the death-domain-containing serine-threonine kinase receptor-interacting kinase (RIP) (Hsu *et al.*, 1996). A complete review of the subsequent molecular interactions that lead to IKK phosphorylation is beyond the scope of this thesis, but briefly it appears that TRAF-2 promotes recruitment of the IKK complex to RIP, which activates the kinases of IKK (Devin *et al.*, 2000). Subsequent phosphorylation of I κ B allows release of NF κ B and unmasking of their nuclear location sequence. Thereafter, translocation of the NF κ B, into the nucleus permits binding of this transcription factor to regulatory sequences of target genes.

The second major transcription factor commonly activated following binding of TNF- α to its receptors is AP-1. As discussed in **Section 1.4.3.2.**, regulation of AP-1 directed transcription of target genes may be regulated in a post-translational manner by differential phosphorylation of its c-Jun and c-Fos components. This is achieved primarily by JNK following ligation of TNFRI by TNF- α . Evidence from studies employing knockout mice and/or mice expressing dominant-negative enzyme forms, suggest that TNF- α -induced activation of JNK, and therefore of c-Jun phosphorylation, occurs via a non-apoptotic TRAF-2 dependent pathway (Lee *et al.*, 1997; Reinhard *et al.*, 1997; Yeh *et al.*, 1997). TRAF-2 appears necessary for coupling the JNK pathway to TNFRI, via MEK7 and MEK4 (Tournier *et al.*, 2001). However, the MEKK linking TRAF-2 and MEK7/4 remains undefined. Potential roles have been postulated for MEKK1 and apoptosis-signal-regulating kinase (ASK)-1 (Baud *et al.*, 1999; Hoeflich *et al.*, 1999), as well for members of the germinal centre kinase (GSK) family (Yuasa *et al.*, 1998)], which are recognised proximal activators of MAPK pathways via phosphorylation of MEKKs (Kyriakis, 1999). These latter kinases may serve to link TNFRI ligation to JNK activation by promoting a functional interaction between TRAF-2 and MEKK1 (Shi *et al.*, 1999). However, unlike the NF κ B pathway, there appears no functional role for RIP, as RIP knockdown fails to inhibit TRAF-2-induced JNK activation (Yuasa *et al.*, 1998).

In contrast, good evidence exists to suggest an axis that TNF-induced activation of the p38 MAPK cascade relies on a functional interaction between the adaptor protein TRAF-2 and RIP (Yuasa *et al.*, 1998), though there is clearly a degree of redundancy in this pathway, as ASK1 and MEKK1 have also been strongly implicated in its activation (Carpentier *et al.*, 1998). Mechanisms underlying ERK1/2 activation following engagement of TNFRI by TNF- α are far less well understood, though the recruitment of a protein containing a low DD homology termed MAPK-activating death domain protein (MADD) to the DD of TNFRI has been implicated (Schievella *et al.*, 1997).

It is clear that signalling mechanisms downstream of TNF- α binding to its receptors is extremely complex, with extensive cross-talk between pro-apoptotic pathways (not discussed in this thesis), NF κ B and MAPK signalling cascades. The molecular basis for this cross talk remains unclear, and aside from cell to cell variation, the importance of signal intensity and duration in promoting differential activation of one of these pathways is only now becoming clear.

1.8.2. The role of TNF- α in lung fibrosis

As alluded to earlier, epithelium-derived TNF- α may be regarded as an endogenous danger signal indicative of cellular injury. This “early wave” alarm-type cytokine is upregulated soon after injury in many tissues, including the lung. Although, haematopoietic cells, especially macrophages, have previously been regarded as the major TNF- α -producing cell, in fibroproliferative lung disease, the epithelium also appears to be a major contributor. Indeed, there exists good evidence that TNF- α plays an important role in the pathogenesis of IPF. Functional TNF- α polymorphisms are associated with an increased risk of developing IPF (Whyte *et al.*, 2000). TNF- α immunoreactivity in IPF lungs is significantly greater than in non-fibrotic control lungs (Kapanci *et al.*, 1995; Nash *et al.*, 1993; Piguet *et al.*, 1993; Ziegenhagen *et al.*, 1998), localising in particular to injured epithelial cells and macrophages. These findings are supported by animal models of lung fibrosis, where increased expression of TNF- α is observed following bleomycin-induced injury (Phan *et al.*, 1992; Piguet *et al.*, 1989), localising to apoptosing epithelial cells (Oikonomou *et al.*, 2006). Interestingly, mice which lack TNF- α expression in haematopoietic cells are not protected from bleomycin-induced fibrosis – in contrast, abolishing TNF- α expression by non- haematopoietic cells results in complete disease protection (Oikonomou *et al.*, 2006). These data are consistent with the hypothesized mechanism of action of bleomycin in this model, which is to induce breaks to double-stranded DNA in epithelial cells – the co-localisation of TNF- α to apoptosing epithelial cells strongly support the notion that epithelium-derived TNF- α has an important role in the pathogenesis of lung fibrosis. In support of a pro-fibrotic role for TNF- α are observations that therapies targeting TNF- α attenuate experimentally-induced fibrosis in animal models (Piguet *et al.*, 1989; Piguet *et al.*, 1994). Moreover, mice deficient in both TNFR1 and TNFR2 are protected from bleomycin-induced fibrosis (Ortiz *et al.*, 1998), though more recent studies have suggested a degree of redundancy in these receptors (Oikonomou *et al.*, 2006).

As discussed in **Section 1.8.1.**, human TNF- α exists in two forms which may have distinct roles. This notion is supported by the observation that mice which express only the transmembrane form (mTNF) are capable of mounting an inflammatory response consisting mainly of macrophages in response to bleomycin. However, these mice are protected from the subsequent development of fibrosis which is associated with a significant impairment in lymphocyte recruitment. Importantly, administration of sTNF- α restores lymphocyte recruitment and susceptibility to fibrosis in this model (Oikonomou *et al.*, 2006). These data suggest that sTNF- α is

required for lymphocyte accumulation and the transition from an inflammatory response to one of fibrosis, and is in accordance with previous studies examining the effect of over-expression of TNF- α in the lung. These mice, over-expressing TNF- α under the control of surfactant protein C, develop a lymphocytic alveolitis associated with collagen deposition over the ensuing months (Miyazaki *et al.*, 1995). Transient over-expression of TNF- α also results in a significant lymphocytosis, with a subsequent fibrotic distortion of lung architecture (Sime *et al.*, 1998), further supporting the hypothesis that TNF- α plays a critical role in lung fibrogenesis.

However, the molecular mechanisms underlying the pro-fibrotic potential of TNF- α are unclear. Indeed, up-regulation of TNF- α expression has been documented in other inflammatory and immune lung pathologies in which normal lung repair processes ensue without evidence of fibrotic reactions, suggesting that TNF- α may be acting via a number of other directly pro-fibrotic cytokines, such as TGF- β . Moreover, it is possible that the pro-fibrotic potential of TNF- α is only realised in the context of a damaged basement membrane, reflecting persistent and repetitive epithelial injury, features felt to be critical to fibroproliferative disease in the lung. In addition, the dysregulated epithelial-mesenchymal crosstalk, characteristic of lung fibrosis, may serve to amplify the pro-fibrotic nature of TNF- α , - these effects may be enhanced in the presence of other pro-fibrotic mediators entrapped within a disordered extracellular matrix (Bringardner *et al.*, 2008).

A common feature of fibrotic disease in animal models where TNF- α is felt to have a prominent role is an inflammatory cell infiltrate, particularly of lymphocytes (Miyazaki *et al.*, 1995; Oikonomou *et al.*, 2006). The mechanisms of this TNF- α -mediated accumulation of inflammatory cells, including lymphocytes, are likely to involve both direct effects of TNF- α itself on regulation of adhesion molecule expression and induction of other cytokines and growth factors capable of mediating leukocyte chemotaxis and survival (Ulrich, 1993). In addition, it appears that expression of TGF- β is upregulated in lung pathology where TNF- α is associated with fibrosis (Phan *et al.*, 1992; Sime *et al.*, 1998). Indeed, TNF- α has been reported to upregulate activity of lung fibroblast-derived-TGF- β *in vitro* both by increasing transcription and stabilising mRNA levels. TNF- α may further promote fibrosis by inducing fibroblast chemotaxis and proliferation, the latter occurring in a PDGF-dependent manner (Battegay *et al.*, 1995; Leibovich *et al.*, 1987).

The potential of TNF- α to act as an endogenous danger signal, promoting maturation and activation of dendritic cells, has previously been documented (Efron

et al., 2005). Furthermore, ample evidence indicates that TNF- α may also directly influence the adaptive immune response. In particular, TNF- α has been shown to enhance T cell proliferation in response to antigens, alloantigens and mitogens (Hackett *et al.*, 1988; Shalaby *et al.*, 1988; Yokota *et al.*, 1988) and in this regard, TNF- α is very similar to IL-1 β . Interestingly, the potential importance of danger signals, including IL1 β and eATP, released from injured epithelium, in the pathogenesis of fibrosis has recently garnered much interest (Gasse *et al.*, 2009; Riteau *et al.*, 2010). One may therefore postulate that in the context of epithelial cell injury, TNF- α represents a danger signal to resident or infiltrating cells, the sequelae of which may be to promote a pro-fibrotic microenvironment. In addition, TNF- α has been demonstrated to play a critical role in the mediation of T-2 cytokine host responses (Artis *et al.*, 1999) and it has been hypothesised that TNF- α functions in this regard through regulation of T-2 cytokine receptor expression (Lugli *et al.*, 1997).

Despite the success of anti-TNF therapy in attenuating experimentally-induced fibrosis, an exploratory trial of etanercept, a soluble decoy TNF- α receptor, in patients with IPF did not demonstrate any significant improvement in primary outcome measures (Raghu *et al.*, 2008). However, this trial was relatively small, compared to recent drug trials in IPF: 88 patients compared to over 700 patients combined in the recent CAPACITY trials evaluating the use of pirfenidone. This small size may have limited the ability of the trial to detect statistically significant differences in the primary end points. Moreover, the non-significant trend to improvement in a number of secondary parameters, including *post hoc* analysis of rate of disease progression by death, may become more apparent with larger patient groups. Furthermore, it is becoming increasingly apparent that IPF is likely to represent the end stage of “multiple hits to multiple pathways”. The success of pirfenidone in reducing the rate of deterioration in lung function in IPF patients reflects this point. Pirfenidone is a pyridine derivative which possesses numerous anti-fibrotic (Gurujeyalakshmi *et al.*, 1999; Hewitson *et al.*, 2001), anti-inflammatory (Oku *et al.*, 2002) and anti-oxidant properties (Giri *et al.*, 1999). With respect to the work presented in this thesis, it is interesting to note that pirfenidone possesses potent anti-TNF- α activity both *in vitro* (Nakazato *et al.*, 2002) and *in vivo* (Iyer *et al.*, 2003; Oku *et al.*, 2002; Oku *et al.*, 2008), as well being capable of attenuating T-2 cytokine expression and airway remodelling in experimentally-induced lung inflammation (Hirano *et al.*, 2006).

The success of future therapeutic strategies in IPF may depend, therefore, on the simultaneous targeting of multiple pathways, the predominance of which may further vary from patient to patient, as well as temporally within an individual. Advances in molecular profiling technologies, including genetic testing and the identification of disease biomarkers, may facilitate a greater degree of personalised drug delivery, thus affording the scientific community an opportunity to make a significant impact in reducing the morbidity and mortality of complex end-stage pathologies such as fibrosis.

1.9. Summary and hypothesis

Pulmonary fibrosis is the end stage of a heterogeneous group of conditions characterised by excessive and disordered ECM deposition within the pulmonary interstitium. Current evidence suggests that defective cross-talk between epithelial cells and mesenchymal cells following repetitive lung injury is a central mechanism contributing to the persistence of activated and highly synthetic myofibroblasts, the key effector cells of fibrogenesis. This defective crosstalk may be further influenced by the immune response to injury and a cytokine milieu dominated by T-2 cytokines is widely regarded as being pro-fibrotic.

Recent evidence has highlighted TSLP as a critical cytokine involved in the polarisation of immune responses towards a T-2 phenotype through its unique activation of dendritic cells as well as direct effects on naïve CD4+ T cells. In addition, recent studies have demonstrated the importance of non-antigenic endogenous danger signals in engaging the adaptive immune response to inflammatory responses following epithelial injury.

Although TSLP has traditionally been regarded as an epithelium-derived cytokine, other potential cellular sources include airway smooth muscle cells. However, the mechanisms underlying TSLP expression by mesenchymal cells remains unclear. Moreover, the role of TSLP in the development of a pro-fibrotic T-2 immune response in non-allergen driven lung disease is unknown.

This thesis will therefore address the following hypothesis:

The induction of TSLP following epithelial lung injury contributes to the development of pulmonary fibrosis by promoting a T-helper 2 immune response

The specific aims of this thesis are to:

1. Examine the immunolocalisation of TSLP and its receptor in human IPF lung tissue and in a murine model of bleomycin-induced lung fibrosis;
2. Determine the role of TNF- α as a danger signal in promoting TSLP expression by alveolar / lung structural cells *in vitro* and *in vivo*;

3. Evaluate the contribution of TSLP to the development of a T-2 immune response following lung epithelial injury;
4. Establish a causal relationship between TSLP and the induction of lung fibrosis following lung epithelial injury.

CHAPTER 2: MATERIALS AND METHODS

MATERIALS

2.1. Chemicals, solvents and tissue culture materials

All chemicals were of analytical grade and were obtained from Sigma Aldrich (UK) unless otherwise stated. All water used for the preparation of buffers was distilled and deionised using a Millipore Water Purification System (Millipore RO10 followed by Milli-Q Plus; Millipore Ltd, UK). Solvents used for the preparation of high pressure liquid chromatography (HPLC) buffers and solutions were of HPLC grade and obtained from BDH-Merck Ltd (UK). Sterile tissue culture flasks and plates, polypropylene centrifuge tubes and pipettes were obtained from Nunc (Denmark). All sterile tissue culture media, sterile tissue culture grade trypsin/EDTA, antibiotics (penicillin/streptomycin) and glutamine were obtained from *Invitrogen (UK)*. Foetal bovine serum (FBS) was obtained from Invitrogen (UK). Dulbecco's Modified Eagle Medium (DMEM) and RPMI-1640 were purchased from Gibco (UK).

2.2 Cytokines

Human recombinant (hr) TNF- α was purchased from Peprotech (NJ, USA) and was dissolved in PBS/0.1% BSA (w/v), aliquoted and stored (1 μ g/ml) at -80°C. hrTSLP, murine TSLP and hrCCL2 were purchased from R&D Systems (UK) and prepared as above. Prior to use, aliquots of appropriate cytokines were thawed and diluted in serum-free medium. Bleomycin was purchased from Kyowa-Hakka (UK) and was dissolved in normal (0.9%) saline prior to use.

2.3. Inhibitors, antibodies and peptides

All the inhibitors used for cell signalling studies were purchased from Calbiochem (USA) unless otherwise stated. All were dissolved in 100% DMSO, aliquoted and stored at -80°C, and were added to cells 30 mins prior to stimulation.

The monoclonal rat anti-mouse TSLP antibody (28F12) was employed for *in vivo* studies in mice to neutralize TSLP, and was a kind gift from GlaxoSmithKline (GSK; UK). The isotype control antibody used in this study was a rat-derived IgG2a targeting the plant protein *Avena phytochrome*, and was also a kind gift from GSK. These antibodies were used at a concentration of 15 mg/kg body weight, and were

dissolved in DPBS. The antibodies use for immunohistochemical localization studies are described in **Table 2.1**. The blocking peptides used for immunohistochemical studies were purchased from Pro-Sci (CA, USA). The antibodies used for Western blotting analysis and flow cytometry are listed in **Table 2.2**. and **Table 2.3**. respectively. All other antibodies used for experimental work are listed in **Table 2.4**.

2.4. Primary cells and cell lines

A human type II alveolar epithelial cell line (A549) and a human monocyte/macrophage cell line (THP-1) were obtained from the American Type Culture Collection (ATCC) and were used at no more than passage 30. Although the A549 cell line was originally obtained through explant culture of lung carcinoma tissue, it is nevertheless widely used as a model of alveolar epithelial cell behaviour as it expresses many characteristic markers of this cell type (Foster *et al.*, 1998; Lieber *et al.*, 1976). For the experimental work described in this thesis, A459 cells were maintained in F12K (Gibco, UK) with 10% FBS (v/v), 200 units/ml penicillin/streptomycin and 4 mM glutamine. The human monocyte/macrophage cell line (THP-1), which was initially derived from a patient with acute monocytic leukaemia, possesses a number of phenotypic features characteristic of human monocytes (Tsuchiya *et al.*, 1980). THP-1 cells were grown and maintained in RPMI-1640 (Gibco, UK) with 10% FBS (v/v), 200 units/ml penicillin/streptomycin and 4 mM glutamine, but were transferred into DMEM for chemotaxis experiments to enable meaningful comparison with conditioned media from treated primary human lung fibroblasts (pHLFs).

Primary human Type II alveolar epithelial cells used in these studies were prepared from normal human lung and were a kind gift from Mr. Robert Alexander in our laboratory.

Fibroblasts used in these studies were primary human lung fibroblasts (pHLFs), and were a kind gift from Dr. Robin McNulty in our laboratory. These fibroblasts had initially been grown from $\sim 1 \text{ mm}^3$ explants dissected from normal human lung tissue. The explants were then cultured in DMEM supplemented with 10% FBS (v/v). The culture medium was replaced with fresh medium one day after isolation, and every three days thereafter for three weeks. Cells were collected by trypsinisation.

Target	Target species	Host species	Company	Secondary (species) - HRP	Company	Primary concentration	Antigen extraction
TSLP	Human	Sheep ¹	R&D	n/a	n/a	2 µg/ml	Citrate
TSLPR	Human	Rabbit	Pro-Sci	Goat	Invitrogen	5 µg/ml	Saponin
TSLP	Mouse	Rabbit	Pro-Sci	Goat	Invitrogen	5 µg/ml	None
TSLPR	Mouse	Rabbit	Pro-Sci	Goat	Invitrogen	0.3125 µg/ml	None
TNF-α	Human	Rabbit	AbCam	Goat	Invitrogen	5 µg/ml	Saponin
CD11c	Human	Rabbit	Epitomics	Goat	Invitrogen	1 in 50	Proteinase K
Isotype control	Human	Sheep	Santa Cruz	n/a	n/a	As per primary	As per primary
Isotype control	Human	Rabbit	Dako	Goat	Invitrogen	As per primary	As per primary
Isotype control	Mouse	Rabbit	Santa Cruz	Goat	Invitrogen	As per primary	As per primary

Table 2.1. Antibodies used for immunohistochemical analysis of human and mouse lung tissue. ¹ *biotinylated antibody, therefore no secondary required.*

Target	Host species	Dilution	Company
Phospho c-Jun	Rabbit	1 in 2000	Cell Signalling Technology MA, USA
Total c-Jun	Rabbit	1 in 2000	Cell Signalling Technology
Phospho ERK1/2	Rabbit	1 in 2000	Cell Signalling Technology
Total ERK1/2	Rabbit	1 in 2000	Cell Signalling Technology
Phospho HSP27	Rabbit	1 in 2000	Cell Signalling Technology
Total HSP27	Mouse	1 in 2000	Cell Signalling Technology
IκB	Rabbit	1 in 2000	Cell Signalling Technology
Phospho STAT3	Rabbit	1 in 2000	Cell Signalling Technology
Total STAT3	Rabbit	1 in 2000	Cell Signalling Technology
Phospho STAT5	Rabbit	1 in 2000	Cell Signalling Technology
Total STAT3	Rabbit	1 in 2000	Cell Signalling Technology
Total ERK2	Goat	1 in 2000	Santa Cruz
Anti-rabbit secondary	Goat	1 in 2000	Dako
Anti-goat secondary	Rabbit	1 in 2000	Dako

Table 2.2. Antibodies used for Western blotting

Target	Target species	Host species	Company	Dilution	Conjugate
CD11c	Mouse	Hamster λ	BD Biosciences	1 in 200	APC Cy7
MHC II	Mouse	Rat κ	eBiosciences	1 in 200	Pacific Blue
CD86	Mouse	Rat κ	BD Biosciences	1 in 200	PE Cy7
TCR β	Mouse	Hamster λ	BD Biosciences	1 in 200	APC Cy7
CD4	Mouse	Rat κ	BD Biosciences	1 in 200	FITC
CD8	Mouse	Rat κ	BD Biosciences	1 in 200	Pacific Blue
IL4	Mouse	Rat κ	BD Biosciences	1 in 200	PE
IFN- γ	Mouse	Rat κ	BD Biosciences	1 in 200	APC
OX40L	Mouse	Rat κ	eBiosciences	1 in 200	PE

Table 2.3. Antibodies used for flow cytometry on lung single cell suspensions.

Chemotaxis assay

Target	Host species	Dilution / Concentration	Company	Secondary species	Dilution	Company
Human CCL2	Goat	Please refer to text for each experiment in Results section	R&D	N/A	N/A	N/A

Immunofluorescence

Target	Target species	Host species	Concentration	Company	Fixation ¹	Secondary antibody	Company	Conjugate
IL7R	Human	Goat	2.5 µg/ml	Santa Cruz	PFA	Donkey	Invitrogen	AF555
TSLPR	Human	Rabbit	2.5 µg/ml	Pro-Sci	PFA	Donkey	Invitrogen	AF488
Isotype control	Human	Goat	2.5 µg/ml	Santa Cruz	PFA	Donkey	Invitrogen	AF555
Isotype control	Human	Rabbit	2.5 µg/ml	Santa Cruz	PFA	Donkey	Invitrogen	AF488

Table 2.4. Antibodies used for chemotaxis assays and immunocytofluorescence. ¹ PFA, paraformaldehyde

Fibroblasts were identified by morphological characterisation, and by differential immunocytochemical staining for a selection of smooth muscle cell, endothelial and fibroblast markers such as α -smooth muscle actin (α -SMA), von Willebrand factor, vimentin and myosin. They were used at no more than passage 7. During the course of this PhD, I contributed to the generation of multiple other such lines of pHLFs. Primary mouse lung fibroblasts (pMLFs) were a kind gift from Dr. Paul Mercer in our laboratory.

METHODS

2.5. Animals

Male C57Bl/6J mice (Charles River, UK) were housed in a specific pathogen-free facility in individually ventilated cages, with free access to food and water (12 hour light/dark cycle, normal sodium dry fishmeal diet, temperature 18-20°C). All procedures were performed on mice between 8 and 12 weeks of age, in accordance with the UK Home Office Scientific Procedures Act (1986). Animals were weighed prior to investigation, and then every few days until the end of the experiment.

2.6. Animal model of pulmonary fibrosis

Bleomycin (2 mg/kg body weight in 50 μ l of saline) or saline was administered by oropharyngeal instillation as previously described (Lakatos *et al.*, 2006). Briefly, following light halothane-induced anaesthesia, mice were hung by their teeth from an elastic band, and had their noses pinched shut while their tongue was held. This prevented the swallow reflex and forced the mouse to breathe through its mouth. Consequently, saline or bleomycin administered to the back of the animal's throat was aspirated. Mice were sacrificed by intraperitoneal injection of phenobarbitone and severing of the abdominal inferior vena cava. For measurement of total lung collagen and real time RT-PCR analysis, lungs were removed and blotted. The trachea and major airways were excised before the separated lobes were immediately snap frozen in liquid nitrogen, weighed and pulverised in liquid nitrogen. Total lung collagen was determined by measuring hydroxyproline content in aliquots of pulverized lung, as described in **Section 2.8**. For RNA extraction, 1 ml of TRIzol (Gibco, UK) was added to a small aliquot of frozen pulverised lung and stored at -80°C until required, as described in **Section 2.9**. For histological and immunohistochemical analysis, the trachea was cannulated and the lungs were insufflated with 4% paraformaldehyde (PFA) in PBS at a pressure of 20 cm H₂O. Thereafter, the heart and lungs were removed *en bloc* and placed in fresh fixative

for 4 hrs. They were subsequently transferred to 15% sucrose in PBS and left overnight, before being washed in 50% ethanol and final transferred to 70% ethanol. For flow cytometric analysis, lungs were placed in RPMI on ice prior to the generation of lung single cell suspensions, as described in **Section 2.10**.

Time-course data for this model was evaluated over a 28 day period. Parameters evaluated included total lung collagen and *Col1A* mRNA levels, and lung sections were prepared for immunohistochemical analysis; for details, please refer to each individual results section as appropriate. Time-points evaluated were days 7, 10, 14, 21 and 28.

2.7. TSLP neutralization *in vivo* using 28F12

The contribution of TSLP to bleomycin-induced polarisation of the immune response and lung collagen accumulation *in vivo* was investigated using a neutralizing antibody to circulating TSLP, 28F12. A prophylactic schedule of administration was employed for each experiment as shown in **Figure 2.1**. Bleomycin or saline was administered by oropharyngeal administration as described in **Section 2.6**. 28F12 or the isotype (IC) control antibody was administered by intraperitoneal injection at a dose of 15 mg/kg body weight in 100 µl DPBS every 4 days, starting at day -1. Serum samples from mice were also taken at the time of sacrifice in the 14 day bleomycin model for measurement of circulating 28F12 and IC antibody concentrations at GSK by ELISA of IgG (**Appendix A1**). Initial mouse numbers and mouse losses are shown in **Figure 2.1**.

2.8. Determination of total lung collagen

Total lung collagen was determined by measuring hydroxyproline (Hyp) content in aliquots of pulverised lung as described previously (Chambers *et al.*, 1994) assuming that lung collagen contains 12.2 % w/w hydroxyproline (Laurent *et al.*, 1981). Hydroxyproline was quantified by reverse-phase high performance liquid chromatography (HPLC) of 7-chloro-4-nitrobenzo-oxa-1,3-diazole (NBD-Cl)-derivatised acid hydrolysates. Secondary amino acids such as Hyp react with NBD-Cl to generate a chromophore with maximum light absorbance at 490 nm. NBD-Cl also reacts with primary amino acids, but these amino acids have only limited absorbance at these wavelengths. In addition, the reactions with Hyp and proline occur one order of magnitude faster than with primary amino acids (Ahnoff *et al.*, 1981), Interference from primary amino acids is therefore minimised by keeping the derivatisation time to 20 mins, a timepoint at which the extent of Hyp derivatisation with

NBD-Cl at 37°C was previously shown to be maximal for up to 20 nmol Hyp (Campa *et al.*, 1990).

2.8.1. Pre-Column Derivatisation

For each sample, approximately 20 mg of lung powder was accurately weighed and hydrolysed in 2 ml of 6 M HCl for 16 hrs at 110 °C in a pyrex tube. Hydrolysates were decolourised with activated charcoal, filtered through a 0.65 µm filter (Millipore Ltd, UK) and diluted 1 in 50. 200 µl aliquots of each hydrolysate were transferred to a microfuge tube and evaporated to dryness under vacuum on a Speedvac (Thermo-Electron Corporation, UK). The residue was re-dissolved in 100 µl HPLC grade water, buffered with 0.45 M potassium tetraborate (pH 9.5) and reacted with 100 µl 36 mM NBD-Cl (in methanol) to a final concentration of 12 mM NBD-Cl. The samples were then incubated in a hot block at 37°C for 20 mins. The reaction was stopped by the addition of 50 µl 1.5 M HCl. At this point, 150 µl of 3.33x Buffer A (**please refer to Table 2.5. for buffer composition**) was also added. Samples were then filtered through an HPLC low dead-volume filter (pore size 0.22 µm, type GV; Millipore Ltd, UK) into a polypropylene tube insert within an Amber Snap Seal vial (Laboratory Sales Ltd., UK). These vials were then loaded onto the HPLC apparatus and the samples were sequentially injected onto the HPLC column and eluted with an acetonitrile gradient as described below.

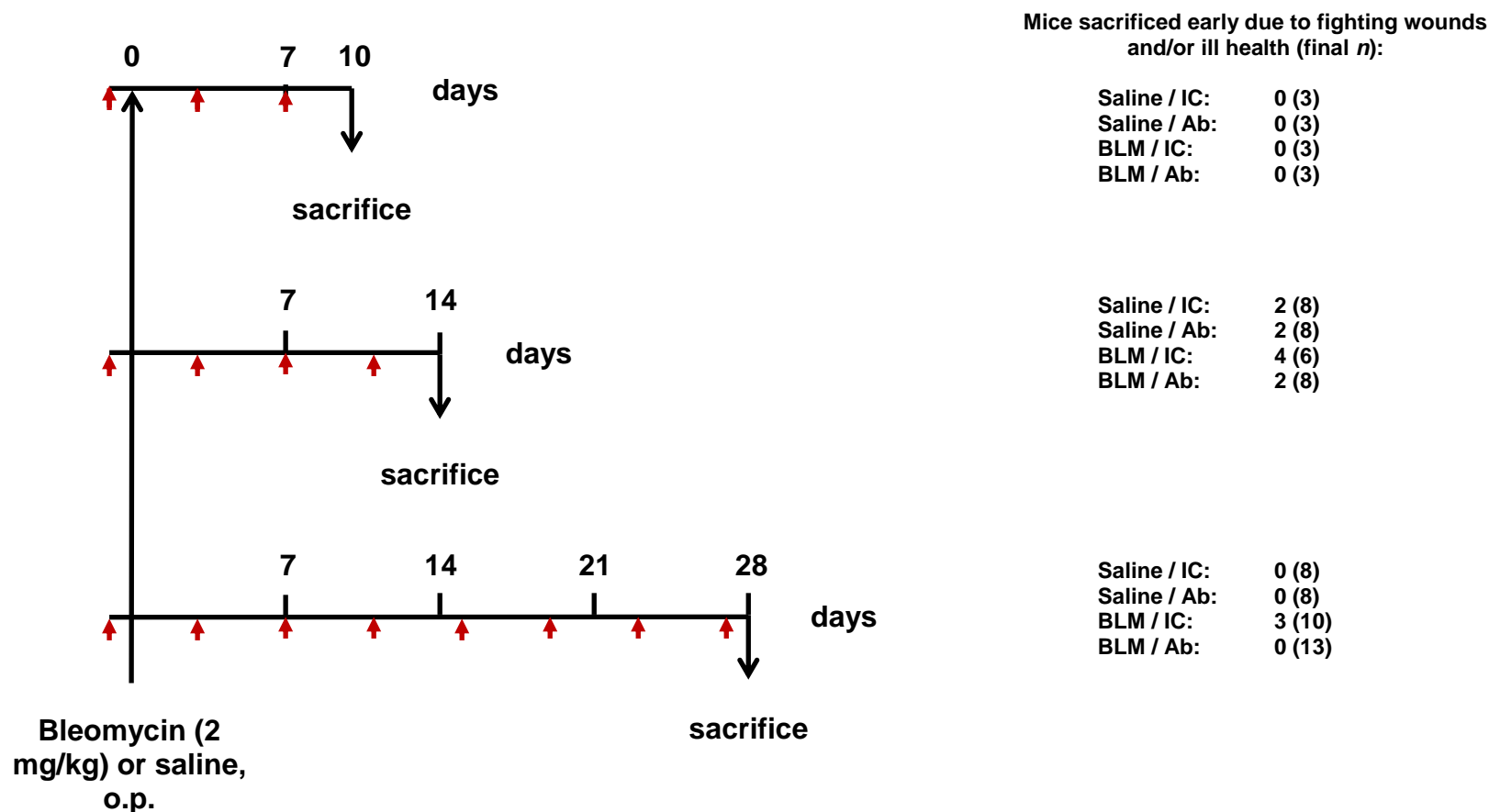


Figure 2.1. TSLP neutralisation *in vivo*. Mice were administered anti-TSLP or isotype control antibody (15 mg/kg body weight) via the intra-peritoneal route every 4 days, starting at day -1 (red dash). Bleomycin (2 mg/kg body weight) or saline was administered at day 0 via the oro-pharyngeal route (o.p.). Mice were sacrificed at day 10, 14 or 28. Mice loss and final numbers are shown on the right. Numbers used for each different analysis are indicated in the relevant figure legend in the **Results** section.

2.8.2. *Instrumentation and chromatography conditions*

The HPLC apparatus employed for these measurements was a Beckman System Gold HPLC (Beckman Coulter, UK) with a reverse-phase cartridge column (LiChroCARTLiChrospher, 250 mm length x 4 mm diameter, 5 µm particle size, 100 RP-18; BDH-Merk, UK) protected by a directly coupled pre-column (LiChrosorb, 4 mm x 4 mm, 5 µm, 100RP-18; BDH/Merck, UK). Columns were continuously maintained at 40 °C in a heated column oven. At the beginning of each experiment, the running buffers were degassed with helium (BOC Ltd, UK), and the HPLC system equilibrated in running buffer A for 40 mins. The first two samples derivatised were Hyp standard solutions (equivalent to 50 pmol Hyp, Sigma, UK) which were then used for calibration. NBD-Cl derivatives in samples and standards were eluted with an acetonitrile gradient, which was achieved by changing the relative proportions of running buffers A and B over time. Chromatographic conditions and buffers employed in this process are summarised in **Table 2.5**.

Post column detection was achieved by monitoring absorbance at 495 nm using a flow through variable wavelength monitor. Hyp elutes as a discrete peak between five and seven mins following its injection onto the column, between glutamine (3.5 mins) and serine (7 to 9 mins), and just prior to the mobile phase becoming organic. Remaining amino acid derivatives in the sample were eluted as the hydrophobicity of the acetonitrile organic buffer was increased. The column running and regeneration time was 25 mins.

Quantification of the hydroxyproline content in each 100 µl sample injected into the column was determined by comparing peak areas of chromatograms obtained for each sample to those generated from the standard solutions derivatised and separated under identical conditions at the beginning of each experiment. Total lung collagen was expressed as mg/lung.

Column	LiChrospher, 100 RP-18, 250 x 4 mm, 5 µm	
Mobile phase	Buffer A – aqueous acetonitrile (8% v/v)	
	50mM sodium acetate, pH 6.4	
	Buffer B - aqueous acetonitrile (75% v/v)	
Column flow rate	1.0 ml/min	
Column temperature	40°C	
Detection wavelength	495 nm	
Elution gradient	Time (min)	% Buffer B
	0	0
	5	5
	6	80
	12	80
	12.5	0
	25	0

Table 2.5. Conditions and buffers for the separation of hydroxyproline by reverse-phase HPLC

2.9. RT-PCR and qRT-PCR analysis

2.9.1. *Precautions taken to prevent RNA degradation*

For all experiments involving RNA isolation, deionised water was pre-treated with 0.1% (v/v) diethylpyrocarbonate (DEPC) overnight at room temperature (RT); the treated water was then autoclaved to inactivate the DEPC. All subsequent reagents were made from molecular biology grade chemicals in DEPC-treated deionised water. All equipment was cleaned thoroughly using RNaseZap (Sigma Aldrich, UK), and nuclease-free pipette tips (Continental Lab Products, UK) were used throughout the extraction process.

2.9.2. *RNA extraction*

Total RNA from frozen lung tissue or cell cultures was isolated with TRIzol reagent as per the manufacturer's protocol (Invitrogen, UK). TRIzol is a solution of phenol and guanidineisothiocyanate which disrupts cell membranes by dissolving cell components but maintaining the integrity of RNA. Briefly, 1 ml of TRIzol was added to 50-100 mg of lung tissue and this was further homogenized through a 25 gauge needle attached to a 1 ml syringe. For isolation from cell cultures, 1 ml of TRIzol was added to each well of cells that were grown in 6 well plates, and the well was scraped with a 1 ml pipette tip before transferring the solution to a 1.5 ml eppendorf tube. Subsequently, samples were left at RT for 5 mins, after which 200 µl of chloroform was added. Each tube was vortexed and left at RT temperature for 10 mins to allow the solution to separate into an upper aqueous and a lower organic phase. Tubes were then centrifuged at 15000 *g* for 15 mins at 4°C in a microfuge, and the upper aqueous phase, containing the RNA, was transferred into a fresh tube with 500µl of isopropanol. Tubes were left at RT for 10 mins to allow the RNA to precipitate, and were then centrifuged at 15000 *g* for 15 mins at 4°C. The supernatants of each tube were discarded and 900 µl of 75% ethanol (BDH VWR International, UK) was added to the pelleted RNA and centrifuged for a further 15 mins at 15000 *g* at 4°C. The supernatants in each tube were discarded and pellets were air dried and resuspended in 12.5 µl of nuclease-free water (Ambion, UK).

2.9.3. DNase treatment

Total RNA was DNase-treated to remove contaminating genomic DNA, using an Ambion DNAfree kit. This kit comprised a 15 µl reaction in which total RNA, DNase I and RNase inhibitor were added and left to incubate at 37°C for 20 mins. The reaction was then stopped by the addition of an inactivating resin which binds the DNase. This resin was subsequently removed by centrifugation.

RNA integrity was assessed by running samples on an agarose gel: a mixture of 1 µl of total RNA and 11 µl DEPC-treated water was mixed with 3 µl of loading buffer (48% deionised formamide (v/v) [Gibco BRL, UK]; 6% formaldehyde (v/v) [BDH, UK]; 5% glycerol (v/v); 20 mM MOPS, 5mM sodium acetate and 1 mM EDTA pH 8.0 made up in DEPC-treated water). The RNA was analysed by electrophoresis on a 1% agarose-formaldehyde gel (6% formaldehyde (v/v); 1% agarose (w/v); 20mM MOPS, 5 mM sodium acetate, 1mM EDTA pH 8.0).

Images of the RNA were captured using a Syngene Gene Genius Bio-imaging system (Synoptics, UK). A ratio of approximately 2:1 of the intensities of 28S rRNA to 18S rRNA bands confirmed that the RNA was not significantly degraded. The RNA concentration and protein contamination was measured on a Nanodrop 8000 spectrophotometer (Thermo Scientific, UK), by measuring the A_{260} and A_{260}/A_{280} ratio respectively. The A_{260}/A_{280} ratio was used as a guide to the purity of the RNA sample; ratios between 1.7 and 2.0 were considered acceptable.

2.9.4. cDNA synthesis

cDNA was prepared by reverse-transcription (RT) using a qScript cDNA SuperMix ® kit (Quanta Biosciences, USA), according to the manufacturer's instructions. Briefly, up to a total of 1 µg of RNA sample was made up to a volume of 16 µl with nuclease-free water. 4 µl of qScript cDNA SuperMix ® (5X reaction buffer containing optimized concentrations of $MgCl_2$, dNTPs [dATP, dCTP, dGTP, dTTP], recombinant RNase inhibitor protein, qScript reverse transcriptase, random primers, oligo(dT) primer and stabilizers) was then added to each sample, to achieve a final volume of 20 µl. Samples were then incubated for 5 mins at 25°C, 30 mins at 42°C and 5 mins at 85°C.

For analysis of *Tslpr* and *Il7r* gene expression studies, PCR products (1 µg) were electrophoresed through a 1% agarose gel containing GelRed (Sigma, UK) diluted

at 1:10,000. Bands were visualised by UV transillumination and the size of the PCR products was estimated using a co-migrated DNA size marker (Roche Diagnostics).

2.9.5. Primer design

The primers were designed in conjunction with Dr. C. Scotton, using internet based software. The EntrezGene (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>) database was used to locate accession numbers for the genomic DNA and RNA and then Spidey (<http://www.ncbi.nlm.nih.gov/spidey/>) was used to align the genomic and RNA sequences in order to locate intron/exon boundaries. Where possible, primers were designed to span introns, eliminating the possibility of amplifying genomic DNA. The mRNA sequence was copied into the primer designing software, Primer 3 (<http://frodo.wi.mit.edu/primer3/>). The program parameters were set at:

Product size	85-130 bp
Primer size	18-22 nucleotides long
Primer melting temperature	58°C – 62°C, optimum temperature 60°C
Max temperature difference	0.5°C
Primer GC %	40-60%, optimum 50%
Max self-complementarity	6.0
Max 3' complementarity	3.00
Max poly-X	3

The primers were then selected from the list. A BLAST search was also performed to check that the forward and reverse primers were specific for the intended sequence (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The primers were manufactured by Invitrogen, UK. The primers used in the present study are listed in **Table 2.6**.

Gene	Forward primer	Reverse primer
<i>hTslp</i>	5'-TATGAGTGGGACCAAAAGTACCG-3'	5'-GGGATTGAAGGTTAGGCTCTGG-3'
<i>hTslp</i> (long splice variant)	5'-GATTACATATATGAGTGGGAC-3'	5'-TTCATTGCCTGAGTAGCAT-3'
<i>hTslp</i> (short splice variant)	5'-CGTAAACTTTGCCGCCTATGA-3'	5'-TTCTTCATTGCCTGAGTAGCATTTAT-3'
<i>hTslpr</i>	5'-GCAAGTCGCTGGATGGTTA-3'	5'-GTCAGAACACGTCACCGTCA-3'
<i>hll7r</i>	5'-GGAGCCAATGACTTTGTGGT-3'	5'-AGTGTGAGCTTTGTGCTGGA-3'
<i>hCcl2</i>	5'-AGCAAGTGTCCTCAAAGAAGC-3'	5'-CATGGAATCCTGAACCCTCT-3'
<i>hHprt</i>	5'-TCATTATGCCGAGGATTTGG-3'	5'-ACAGAGGGCCACAATGTGATGTTG-3'
<i>mTslpr</i>	5'-GCTCCTTCCCTGGACTCTTT-3'	5'-TCCACCCTCTTAGCCTTGG-3'
<i>mll7r</i>	5'-CTTCTGGAGCGAGTGGAGTC-3'	5'-TGACACTTGGCAAGACAGGA-3'
<i>mCol1A1</i>	5'-TCATGGCTTCTCTGGTCTC-3'	5'-CCGTTGAGTCCGTCTTTGC-3'
<i>mHprt</i>	5'-TCATTATGCCGAGGATTTGG-3'	5'-ACAGAGGGCCACAATGTGATGTTG-3'

Table 2.6. Primers used for qRT-PCR

h=human, m=murine

2.9.6. Real-time RT-PCR

Real time RT-PCR was conducted using the Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, UK) with cDNA samples diluted 1:3 or 1:4 in nuclease free water, and forward and reverse primers each at a final concentration of 800 nM (except *mCo/1A1* which was used at final concentrations of 400 nM), on a Mastercycler EP Realplex (Eppendorf, Germany). Cycling conditions were as follows: activation step of 95°C for 10 mins; 40 cycles of 95°C (10 seconds), 62°C (45 seconds), followed by melting curve analysis.

The efficiency of each primer pair was also assessed by determining the crossing threshold (Ct) values for RT-PCR reactions using a series of 1:2 dilutions of template cDNA. Ct values were defined as the earliest point of the linear region of the logarithmic amplification plot reaching a threshold level of detection. Concentrations of samples were plotted against Ct values, and the slope of the plot determined, to determine the efficiency of the primers. Primers were only used if the PCR efficiency was greater than 95%.

To examine the quantitative differences in target mRNA expression in each sample, Ct values were determined from the linear region of the logarithmic amplification plot. Each sample was also tested for the expression of the housekeeping gene, HPRT – the Ct value of which was used to normalise between samples. Fold changes were subsequently calculated using the standard $2^{-\Delta\Delta C_t}$ approach and expressed relative to i) time 0 controls for time course studies *in vitro*, or ii) saline/isotype control for *in vivo* studies. The specificity of the products obtained by PCR was confirmed by analysis of the melting curves. A single melting curve was indicative of a single PCR product. Statistical analysis was performed using the ΔC_t values.

2.10. Flow cytometry

2.10.1. Lung single cell suspension

Following sacrifice of mice, as described in **Section 2.6.**, the trachea and major airways were excised and lobes separated before being placed in RPMI-1640 (Gibco, UK) on ice. Concomitantly, spleens were also harvested from each mouse, and similarly placed in RPMI-1640 on ice. Lungs were minced finely with scissors and incubated in RPMI-1640 containing Liberase TM (Roche, UK) and DNase (Roche, UK) at final concentrations of 0.15 mg/ml and 0.25 mg/ml respectively, for 30 mins at 37°C. After digestion, single cell suspensions were generated by filtering

the digested lung through a 70 μ m cell strainer (BD Biosciences, UK). For the generation of splenic single cell suspensions, no digestion was required and whole spleens were teased through a 70 μ m cell strainer. All suspensions were spun at 300 *g* for 5 mins, and cell pellets were resuspended in 1 ml of red blood cell lysis buffer (RBCLB) (Sigma, UK) and incubated at RT for 5 mins. Suspensions were then made up to a final volume of 15 ml with RPMI-1640 and spun at 300 *g* for 5 mins. Cell pellets were resuspended in PBS/0.2% BSA (w/v) and used for experiments.

2.10.2. FACS analysis

Positive staining was confirmed by comparison with fluorescence minus one (FMO) controls for each fluorescence channel of interest. For unstained controls, single stain controls and FMO controls, cells were pooled from lungs or spleens as appropriate. The fluorochrome-conjugated antibodies used to characterise mouse lung dendritic cells and lymphocytes, together with their concentrations, are listed in **Table 2.3**.

Prior to antibody staining of samples, non-specific antibody binding was blocked by pre-incubation with rat anti-mouse CD16/CD32 (1 in 200 in PBS/0.2% BSA [w/v]) for 10 mins at RT. For staining of cell surface antigens, cells were incubated with antibody at 4 °C for 30 mins, before being washed in PBS/0.2% BSA (w/v) and fixed in 4% paraformaldehyde for 20 mins at RT. Samples were then stored at 4°C until analysed. For staining of intracellular cytokines, cell suspensions were incubated with phorbol myristate acetate (PMA), ionomycin and Brefeldin A (all Sigma, UK) in RPMI-1640 at final concentrations of 50 ng/ml, 500 ng/ml and 10 μ g/ml respectively for 4 hrs at 37 °C. Thereafter, samples were washed in PBS/0.1% BSA (w/v)/0.1% sodium azide (w/v) (PBA) and incubated with antibodies targeted against cell surface antigens for 30 mins at 4°C. Cells were then washed in PBS and fixed in 2% PFA in hypertonic PBS (x1.4 PBS in ddH₂O) and allowed to stand at RT for 20 mins. After washing in PBA, cells were incubated in PBS/0.1% BSA (w/v) / 0.1% sodium azide (w/v) / 0.5% saponin (w/v) (saponin buffer) for 10 mins at RT. After permeabilisation, antibodies directed against intracellular cytokines were added, and samples were incubated for 30 mins at RT. Samples were washed in saponin buffer and then PBS/0.2% BSA (w/v), before being resuspended in PBS and stored at 4°C for analysis the following day. Control samples for intracellular cytokine analysis were treated in exactly the same manner to ensure comparable forward and side scatter characteristics.

Dendritic cells were identified by the expression of CD11c and MHC II molecules. Alveolar macrophages also express CD11c, but can be distinguished from DCs by:

- 1) High level of autofluorescence
- 2) Low level of MHC II expression

To differentiate macrophages from DCs, analysis was initially undertaken with appropriate gating according to forward and side scatter to exclude lymphocytes and dead cells. Highly autofluorescent cells corresponding to alveolar and interstitial macrophages were then excluded. Using this strategy, cells subsequently identified as being CD11c⁺/MHC II^{hi} cells were considered DCs, as shown in **Figure 2.2**.

T-lymphocytes were identified by the expression of CD4, CD8 and TCR- β . Analysis was initially undertaken with appropriate gating according to forward and side scatter to identify lymphocytes, as shown in **Figure 2.3**. Cells expressing CD4 and TCR- β were considered CD4⁺ T-lymphocytes and used for analysis of intracellular cytokine staining of IL-4 and IFN- γ , markers of Th2 and Th1 lymphocytes respectively.

Data was acquired on a LSR II or LSR Fortessa (BD Biosciences, UK) and analysed using FloJo (TreeStar, USA). At least 30000 events were recorded for each sample. Fluorescence compensation settings for the above multi-colour flow cytometry was achieved using anti-rat IgGk and anti-mouse IgGk compensation bead kits, as per manufacturer's instructions (BD Biosciences, UK). Anti-hamster compensation beads (Invitrogen, UK) were used where appropriate, according to the manufacturer's instructions. Compensation adjustments were made using FlowJo software (TreeStar, UK).

2.11. Histological analysis

2.11.1. *Preparation of slides*

Fresh human lung biopsy material was fixed in 4% PFA for 4 hrs before transfer to 15% sucrose in PBS overnight. After being washed in 50% ethanol, specimens

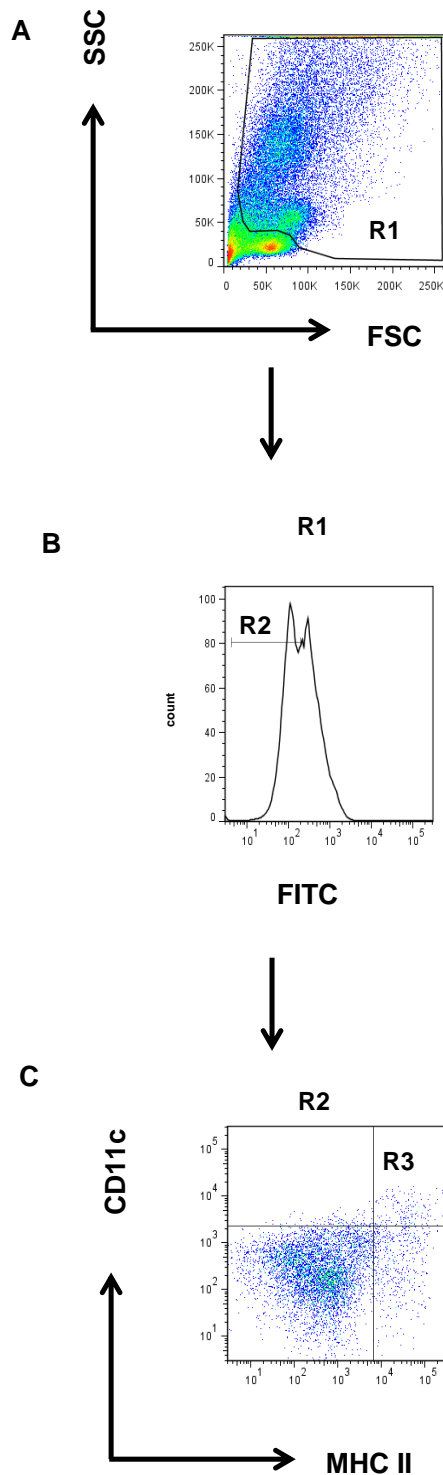


Figure 2.2. Gating strategy employed to identify dendritic cells within lung single cell suspensions.

Viable cells, excluding lymphocytes, were initially gated on forward and side scatter (A). Cells of high auto-fluorescence in the FITC channel, corresponding to alveolar macrophages, were excluded (B) and dendritic cells were identified on the basis of being CD11c⁺ / MHC II^{high} (C). Subsequent analysis for DC surface markers, including CD86 and OX40L, was performed on this cell population (R3).

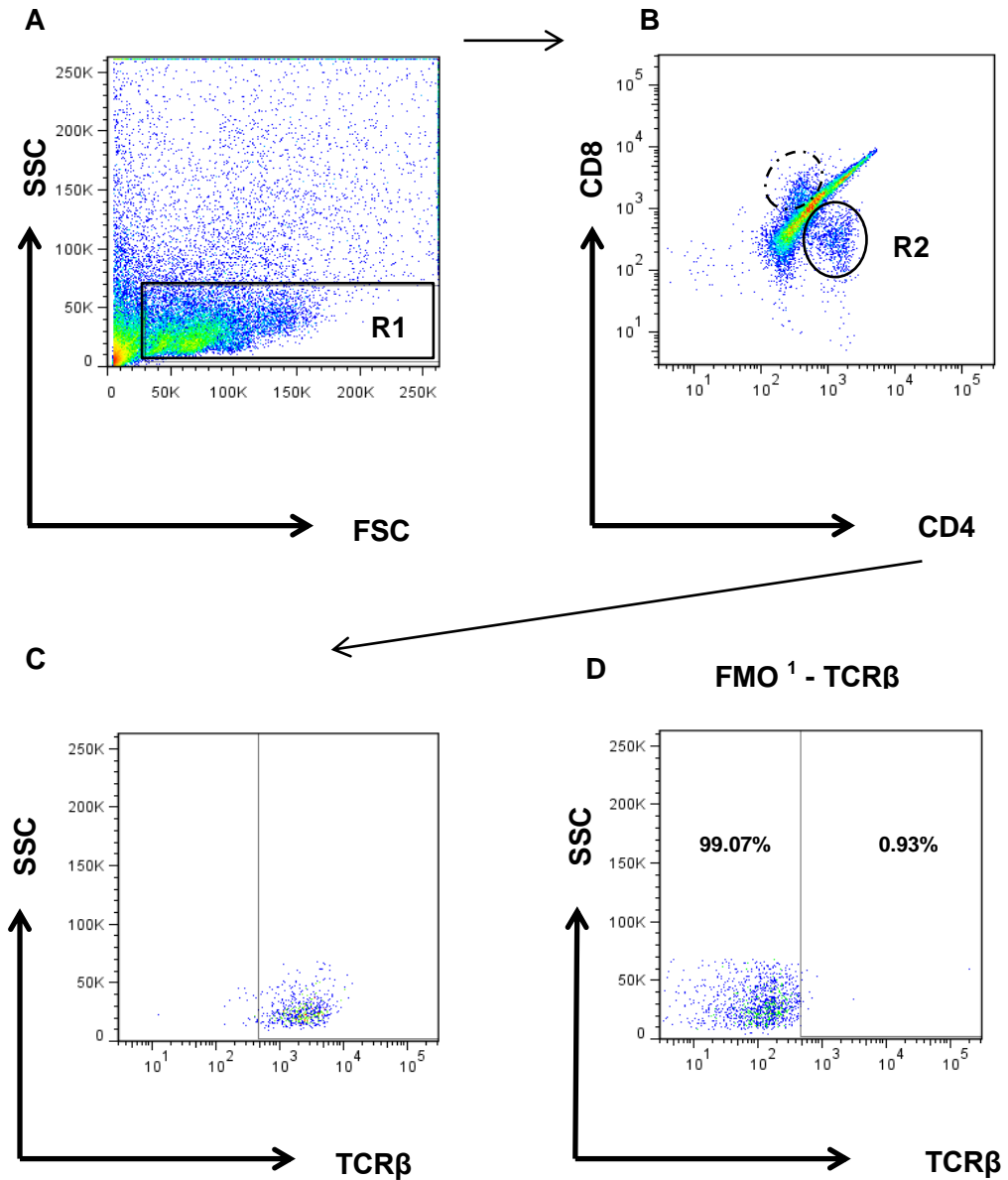


Figure 2.3 Gating strategy employed to identify CD4⁺/CD8⁺ T-lymphocytes within lung single cell suspensions.

Lymphocytes were initially gated by forward and side scatter (**A**). T cells were labelled with anti-CD4, anti-CD8 and TCR-β. All cells identified as CD4⁺ (R2) or CD8⁺ (dashed gate) (**B**) were further identified as being TCR-β⁺ (**C**). Only data for CD4⁺ cells are shown for clarity; CD8⁺ cells were analysed in the same manner. Subsequent analysis for intracellular cytokine staining was performed on this subset of CD4⁺ TCR-β⁺ lymphocytes. ¹ FMO – fluorescence minus one was used as a negative control (**D**).

were transferred to 70% ethanol until required. Individual lobes of fixed mouse lungs or human biopsy material were placed in processing cassettes and dehydrated through a serial alcohol gradient on an automated tissue processor (Leica TP1050, Leica Microsystems, UK) before being manually embedded in paraffin wax blocks. Control lung sections for human TSLP and TSLPR immunostaining were cut from lung resection samples from patients with bronchial malignancies with no parenchymal fibrotic disease. Before immunostaining, 3 µm thick lung sections were dewaxed in xylene, rehydrated through decreasing concentrations of ethanol, and washed in TBS.

2.11.2. *Immunohistochemical localisation of TSLP and TSLPR*

For each antigen, four techniques of antigen extraction were performed to determine the optimum method of antigen retrieval for future studies: washing in TBS (2 x 5 mins); microwaving sections in 10 mM citrate buffer, pH 6.0 (2 x 10 mins); incubation with 0.05% saponin (w/v) (Sigma, UK) in double-distilled water for 30 mins at RT; incubation with proteinase K (10 µg/ml in 5 mM EDTA / 10 mM Tris, pH 7.4) for 10 mins. Human TSLP was unmasked by microwaving sections in 10 mM citrate buffer, as described above. Human TSLPR required incubation with 0.05% saponin (w/v), as described above. Murine TSLP and TSLPR did not require any antigen unmasking.

After antigen retrieval, sections were washed twice in TBS for 5 mins, before incubation in 10% hydrogen peroxide (v/v) in H₂O for 30 mins. Sections were washed again in TBS twice for 5 mins, and then incubated in 1:6 serum (dependent upon which species the secondary antibody had been raised in) in TBS, with 4 drops/ml avidin blocking solution (Vector Laboratories, UK) for 20 mins. Thereafter, the blocking solution was tapped off, and the sections were incubated with the primary non biotinylated polyclonal antibody diluted in TBS/1% BSA (w/v)/1% serum (v/v) as described above and 4 drops/ml biotin blocking solution (Vector Laboratories, UK), overnight at 4°C. Sections were then washed in TBS (2 x 5 mins) before being incubated for 1 hour at RT with a biotinylated secondary antibody diluted 1:200 in TBS/1% BSA (w/v). Sections were again washed in TBS as described above, and then incubated with a streptavidin/peroxidase complex (Invitrogen, UK; 1:200 in TBS) for 30 mins. Sections were then washed in TBS (3 x 5 mins) before a solution of 600 µg/ml of 3,3'-diaminobenzidine tetrachloride (Vector Laboratories, UK) was added for 10 mins for colour development. Thereafter, sections were counterstained using Gill-2 haematoxylin (Thermo Shandon, USA),

dehydrated through a series of incubations in increasing concentrations of ethanol and then xylene, and mounted using a Sakura Coveraid automatic cover-slipping machine and Tissue-Tek cover-slipping film (Bayer Diagnostics, UK). Specificity of the signal obtained was confirmed by incubating control sections with an isotype specific, non-immune primary antibody. Further signal specificity was confirmed where possible by pre-incubating the primary antibody with a blocking peptide (at a concentration 6-fold greater than that of the primary antibody) overnight at 4°C. For biotinylated primary antibodies, sections were incubated with avidin for 15 mins, washed in TBS (1 x 5 mins) and then incubated in biotin for 15 mins before being washed in TBS (1 x 5 mins) prior to incubation with the primary antibody. No secondary antibody was required in this case. The detection antibody for human TSLP was a biotinylated primary antibody; all antibodies used were non-biotinylated antibodies. Sections were then visualized by microscopy (Leica DM500B microscope, Leica Microsystems, Germany) and images were captured using a Qiacam 12-bit colour fast camera using Q Capture software version 2.81 (QImaging Corporation, Canada).

Optimum concentrations for primary antibodies are described in **Table 2.1**.

2.11.3. *Patient samples*

Lung biopsy specimens were obtained from 12 patients with IPF (obtained at diagnostic surgical lung biopsy) and 3 control patients (obtained from uninvolved tissue during cancer resection surgery). All biopsies used in this study were classified using the diagnostic criteria of the American Thoracic Society/European Respiratory Society Consensus (American Thoracic Society/European Respiratory Society International multidisciplinary consensus classification of the idiopathic interstitial pneumonias, 2002) demonstrating a pattern of usual interstitial pneumonia (UIP). Approval for the use of the material was obtained from the Royal Brompton, Harefield, NHLI and the UCL/UCLH ethic committee. Informed consent was obtained from each patient.

2.12. Cell biology

2.12.1. *Cell culture conditions*

All cells used for experimental work described in this thesis were maintained using standard cell culture techniques. pHLFs and pMLFs were grown in Dulbecco's modified eagle's medium (DMEM), supplemented with penicillin (200 units/ml), streptomycin (200 units/ml), glutamine (4 mM) and 10% (v/v) FBS. pHLFs used in siRNA transfection experiments were grown in media with no antibiotics to improve transfection efficiency. Cells were incubated in a humidified atmosphere containing 10% CO₂. The growth and maintenance of A549 and THP-1 cells has been described in **Section 2.4**. Primary Type II alveolar epithelial cells were seeded following preparation in DCCM-1 (Geneflow, UK) supplemented with penicillin (200 units/ml), streptomycin (200 units/ml), glutamine (4 mM) and 10% (v/v) FBS, and used immediately for experimentation. Cells were routinely tested for mycoplasma contamination using a commercially available assay (MycoAlert; Cambre, UK).

Upon reaching visual confluence (3-4 days), cells were sub-cultured (passaged) into new flasks: the medium was removed and the cell layer was washed with 0.05% trypsin (w/v)/0.02% EDTA (w/v) solution to remove any remaining culture medium. Cells were brought into suspension by adding 5mls of 0.05% trypsin (w/v)/0.02% EDTA (w/v) solution (for a T175 flask), and incubating the cells at 37°C until cell detachment was observed. Trypsin was neutralized by the addition of 15 mls of culture medium supplemented with 10% FBS. Cell suspensions were split at a ratio of 1 to 4-8. pHLFs and pMLFs were used at no more than passage 7 – cells of greater passage were observed to display characteristics of differentiation into myofibroblasts, thus precluding their use in the current studies.

2.12.2. *Preparation of agonists and inhibitors*

Preparation of hrTNF- α , hrTSLP, hrCCL2 and murine TSLP was as described in **Section 2.2**. All inhibitors were reconstituted in sterile tissue culture-tested DMSO (Invitrogen, UK) and stored at -80°C until use. Prior to use, the inhibitors were diluted in serum-free medium to achieve a final experimental DMSO concentration of 0.1%. For cell signalling studies, pHLFs were incubated with the inhibitor for 30 mins at 37°C in 10% CO₂, prior to stimulation with TNF- α .

2.12.3. Cell preparation for experiments

A suspension of pHLFs trypsinised from one tissue culture flask was placed in a 50 ml sterile polypropylene centrifuge tube. Cells were centrifuged at 300 g for 5 mins at RT using a bench centrifuge (MSE Mistral 3000, UK). The supernatant was discarded, and the cell pellet was brought into a single cell suspension with 10 ml DMEM/10% FBS (v/v) by gentle mixing. An aliquot of 7 μ l of the suspension was removed under sterile conditions, and cells were counted on a haemocytometer. For each experiment, appropriate cell density of the cell suspension was then adjusted with DMEM/10% FBS (v/v). For collection of conditioned media for detection of secreted protein by ELISA, pHLFs were seeded at 1×10^5 cells/ml in 100 μ l in 96 well plates. For collection of mRNA in TRIzol, cells were seeded at 1×10^4 cells/ml in 2 ml, in 6 well plates. For collection of cell lysates for Western blotting analysis, cells were seeded at 7.5×10^4 cells/ml in 1 ml in 12 well plates. Please refer to **Section 2.14.** for seeding densities for siRNA transfection experiments. Cells were seeded 48 hrs before assay. After 24 hrs in culture, and before confluence, cells were washed twice with serum-free DMEM, and the cells were quiesced in serum-free DMEM. Before each experiment, the starvation medium was removed and replaced with fresh serum-free DMEM, with or without mediators or inhibitors for a selected length of time (for details, please refer to individual experiments described in **Results** section). Plates were incubated in a humidified atmosphere containing 10% CO₂ at 37°C. For cytokine assays, serum-free conditioned media were collected from cell culture plates, spun to remove cell debris, and either used for ELISA immediately, or stored at -80°C and defrosted on ice before ELISA. A549 cells were treated in the same manner except that cells were incubated in 5% CO₂ in F12K.

2.13. Western blotting

Western blotting was used to evaluate expression of proteins of interest. Samples were solubilised with detergents and reducing agents and separation of proteins from crude lysate was achieved by SDS-PAGE. Following transfer onto nitrocellulose membrane (GE Healthcare, UK), proteins were detected by enhanced chemiluminescence and quantified by using densitometry. The degree of protein phosphorylation was determined using specific antibodies directed against the phosphorylated form of the target protein.

2.13.1. Buffers

Lysis buffer:	Phosphosafe (VWR, UK) Complete mini tablet protease inhibitor (Roche, UK) – 1 in 20 dilution
Laemmli buffer:	0.4M Tris pH 6.8; 12.5% SDS (w/v); 0.25 M glycerol; 0.125 M DTT; bromophenol blue to colour;
Running buffer (x10):	1M Tris base; 1M HEPES; 0.1% SDS (w/v);
Transfer buffer (x10):	1M glycine; 0.125M Tris base; 0.375% SDS (w/v);
Wash buffer (x20):	0.2M Tris base; 0.2M Tris HCl; 2.7M NaCl Used as x1 TBS / 0.1% Tween 20 (TBST)
Blocking buffer:	TBST / 5% w/v non-fat milk powder
Stripping buffer:	62.5M Tris-HCl, pH 6.8; 2% SDS (w/v); 6.25 mM DTT

2.13.2. Preparation of cell extracts

pHLFs were seeded 48 hrs prior to treatment in 12 well plates. Cells were serum-starved for 24 hrs before each treatment. After each treatment, cells were washed twice in ice-cold PBS on ice. Ice-cold lysis buffer (50 µl) was added to each well, on ice, and lysates were scraped and collected into ice-cold tubes. Whole cell extracts were stored at -80°C before use.

2.13.3. BCA protein assay

Protein concentrations of samples were assessed using the bicinchoninic acid (BCA) protein assay (Pierce, USA), as per the manufacturer's instructions. Prior to use, an aliquot of BSA was thawed and diluted in serum-free medium. The amount of protein in samples can be derived by comparison with a bovine serum albumin standard curve. Briefly, 100 µl of freshly prepared BCA working reagent was added to 10 µl of each sample or standard (assayed in duplicate) in a 96 well plate. The plate was incubated at 37°C for 30 mins prior to reading the absorbance at 550nm on a Titertek Multiscan MCC/340 plate reader (Labsystems, Finland). Protein concentrations were determined by comparison with a standard curve of BSA ranging from 0-2000 µg/ml.

2.13.4. *Separation of proteins by reducing SDS-polyacrylamide gel electrophoresis*

Pre-cast LongLife gradient gels (8-16%; Nusep, Appleton Woods, UK) were used for all studies. Samples were mixed with Laemmli buffer and incubated at 100°C for 5 mins before being loaded on to the SDS-PAGE gel. A pre-stained molecular mass ladder (PageRuler Plus, ThermoFisher, USA) was run in a separate lane for identification of appropriate molecular masses. Samples were electrophoresed at 140 V for 40 mins in Nusep running buffer (Nusep, Appleton Woods, UK).

2.13.5. *Transfer of proteins onto nitrocellulose membrane*

Resolved proteins were transferred to a nitrocellulose membrane using a horizontal semi-dry transfer method. The nitrocellulose membrane was pre-equilibrated in transfer buffer for 15 mins prior to blotting. All other components were also soaked in transfer buffer before use. The membrane was placed on top of 10 pieces of Whatmann 3 mm filter paper and overlaid with a gel, taking care to remove air bubbles. A further 10 pieces of filter paper were placed on top of the gel. The cassette assembly was placed into the semi-dry transfer apparatus (Invitrogen, UK) and a constant voltage of 20 V was applied for 1 hour. Membranes were incubated in 2% Ponceau Red solution (Sigma, UK) solution to verify protein transfer.

2.13.6. *Detection of proteins by ECL*

Following transfer, the membrane was incubated in blocking buffer for at least 1 hour with gentle agitation. The membrane was then incubated overnight at 4°C in blocking buffer containing an appropriate dilution of primary antibody. Thereafter, membranes were subjected to three 5 minute washes with wash buffer before incubation with HRP-conjugated secondary antibody, appropriately diluted in blocking buffer. After a further 3 washes, blots were developed by enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham, UK). Immunoreactive bands were visualised by exposing Hyperfilm (GE Healthcare, UK) to the membrane. Exposure time was adjusted accordingly to ensure accurate quantification of protein bands. Films were then developed in an autoradiography film developer (Photon Imaging System, UK). Please refer to **Table 2.2.** for a full list of antibodies used for Western blotting in these studies. To confirm equal loading, membranes were incubated in stripping buffer for 20 mins at 50°C, followed by incubation in blocking buffer for 45 mins. They were then re-probed with either antibodies targeted against the non-phosphorylated form of the target protein or, in the case of siRNA transfection experiments, the housekeeping protein, total ERK2.

2.13.7. Quantification of protein bands

Semi-quantitative analysis of Western blots was performed using densitometry. The blots underwent transmissive greyscale scanning at 300 dpi on a transmissive flatbed scanner (Epson, UK). The optical density of each band was calculated in Image J software (National Institutes of Health, USA) with reference to a calibration curve generated by scanning a Kodak photographic Step Tablet (Kodak, UK) with a known optical density gradient, using the same settings as described above. The optical density of the band for the target protein was normalised to the optical density of the non-phosphorylated form of the same protein, or total ERK2 for siRNA transfection experiments.

2.14. Transfection of pHLFs with siRNA

Primary human lung fibroblasts were seeded into 12 well plates on day 1 at a density such that 80% confluence would be reached by day 5 (3×10^4 cells/ml in 1 ml). Antibiotics were omitted from all cultures involved in siRNA experiments to maximise transfection efficiency. On day 2, cells were serum-starved. Twenty four hrs later, cells were transfected with siRNA directed against either c-Jun, STAT3 or control scrambled siRNA at final concentrations of 100 nM. Further control cells were mock-transfected at the same time. siRNA (Dharmacon, USA) was transfected into cells using a gemini transfection reagent (a kind gift from GSK) in OptiMem medium. Gemini surfactants are amphiphilic molecules which bind and compact dsRNA efficiently, resulting in the creation of a stable lipoplex. This is then able to penetrate the cell membrane into the cytoplasm within endosomes. Escape from the endosome, which is key for successful transfection, ensues as the pH falls within the cells, compared to the extracellular medium. This transfection reagent has previously been used successfully in the host laboratory. Cells were again serum-starved on day 4 and allowed to quiesce for 24 hrs before subsequent stimulation. After each specific treatment, cell extracts were prepared as described in **Section 2.13.2.**, and Western blotting was used to evaluate expression of proteins of interest. In addition, time-point matched conditioned media were collected and analysed for TSLP and CCL2 protein release by ELISA, as described in **Sections 2.15. and 2.16.** respectively.

siRNA sequences for c-Jun and STAT3 are listed below in **Table 2.7.**

Target	Sequence
c-Jun	GAGCGGACCUUAUGGCUAC
STAT3	CAACAGAUUGCCUGCAUUG
Scrambled controls	Sequence not provided by Dharmacon

Table 2.7.

Sequences of siRNA used for transfection of pHLFs.

2.15. Human TSLP ELISA

Buffers used: PBS; Wash buffer: 0.05% Tween 20 ® in PBS; Reagent diluent: PBS/1% BSA (w/v); Stop solution: 1M H₂SO₄; Substrate solution: Tetramethylbenzidine (TMB; Invitrogen, UK)

ELISA: Microtest TM 96-well ELISA plates were obtained from BD Biosciences, USA. The human TSLP DuoSet ELISA set was purchased from R&D Systems, UK. The protocol followed was identical to that in the accompanying literature. Briefly, the Microtest TM 96-well ELISA plates were coated with 50 µl of anti-human TSLP capture antibody (800 ng/ml) diluted in PBS and were then left overnight at 4°C. After overnight incubation, plates were washed using an automated plate washer (Denley, UK) and blocked with 150 µl of reagent diluent for at least 1 hour at RT. After incubation, plates were again washed, and 50 µl of each sample or serial dilutions (0-2000 pg/ml) of human TSLP standard, diluted in serum-free DMEM, were added to each well. After incubation for 2 hrs at RT, plates were again washed, before 50 µl of anti-human TSLP detection antibody (400 ng/ml) diluted in reagent diluent, was added to each well. Following a further incubation at RT for 2 hrs, plates were again washed, before the addition of streptavidin-HRP (1:200) in reagent diluent. Plates were incubated for 30 mins at RT before a further wash. 50 µl of substrate solution was then added to each well and incubated for 30 min prior to the addition of stop solution. Absorbances were then read at 450 nm

and 540 nm with a plate reader (Multiskan MCC/340, Titertek) and TSLP concentrations were determined by comparison with a standard curve of TSLP ranging from 15 pg/ml to 2000 pg/ml.

2.16. Human CCL2 ELISA

Buffers used: Bicarbonate buffer (pH 9.6): 1.59 g Na₂CO₃ and 0.2 g NaN₃ in 1L double-distilled water, filtered and stored at RT; Blocking buffer: PBS/1% BSA (w/v); Wash buffer: 0.05% Tween 20® in PBS; Stop solution: 1M H₂SO₄.

ELISA: ELISA plates were coated with anti-human CCL2 capture antibody (400 ng/ml in bicarbonate buffer), 50 µl per well, and incubated overnight at 4°C. Plates were then washed as described above, and blocked with 150 µl of blocking buffer for at least 1 hour at RT. After incubation, plates were again washed, and 50 µl of each sample (diluted 1 in 10) or serial dilutions (0-625 pg/ml) of human CCL2 standard, diluted in serum-free DMEM, were added to each well. After incubation for 2 hrs at RT, plates were washed before 50 µl of anti-human CCL2 detection antibody (400 ng/ml in blocking buffer), was added to each well. Following a further incubation at RT for 2 hrs, plates were again washed, before the addition of streptavidin-HRP (diluted 1:200 in PBS/0.1% BSA [w/v]). Plates were incubated for 30 mins at RT before a further wash. 50 µl of substrate solution was then added to each well and incubated for 30 min prior to the addition of stop solution. Absorbances were then read at 450 nm and 540 nm with a plate reader (Multiskan MCC/340, Titertek) and CCL2 concentrations were determined by comparison with a standard curve of CCL2 ranging from 19.5 pg/ml to 625 pg/ml.

2.17. Monocyte chemotaxis

Chemotaxis of the human monocyte/macrophage cell line, THP-1, was evaluated in a 48-well Boyden chamber (Neuroprobe, USA). The ability of conditioned media from pHLFs to induce chemotaxis of THP-1 cells was compared to control medium alone and human recombinant CCL2, in the presence or absence of a neutralising anti-CCL2 antibody or isotype control antibody. Lower wells of the chamber were filled with 28 µl of chemotactic agent, controls or conditioned media. A polyvinylpyrrolidone (PVP) treated polycarbonate filter (8 µm pore size) (Neuroprobe, USA) was lowered onto the lower chambers, taking care not to introduce air bubbles, and a silicone gasket was applied to the filter, upon which the upper chambers would ultimately be positioned. THP-1 cells were prepared in

DMEM/10% FBS (v/v) at a concentration of 1×10^6 cells/ml, and 50 μ l were added to the upper chambers, again taking great care not to introduce air bubbles. The chamber was then incubated at 37°C in humidified air with 5% CO₂ for 2 hrs. After incubation, the filter was removed and non-migrated cells on the upper side of the filter were wiped clean using a wiper blade. The filter was then fixed in methanol, before being stained in Diff-Quik (Thermo Scientific, UK). The filter was then placed on a clear microscopic slide and allowed to dry, before migrated cells were counted. The mean number of cells per 5 high powered fields was determined, and each experimental condition was performed in triplicate. Each experiment was performed on three separate occasions.

2.18. Immunocytofluorescence

Immunocytofluorescence staining was performed to identify TSLPR and IL7R α receptor chains on pHLFs grown *in vitro*. pHLFs were seeded in 8-well chamber slides (Millipore, UK) at 2×10^6 cells/ml in 250 μ l DMEM and left overnight to adhere in a humidified atmosphere with 10% CO₂ at 37°C. After 24 hrs, cells had reached semi-confluence, and were washed gently with PBS. Thereafter, cells were fixed with 4% PFA in PBS for fifteen mins. Following fixation, cells were washed with PBS three times. To avoid non-specific binding, cells were incubated in blocking buffer (PBS/10% FBS (v/v)/0.2% fish skin gelatin (v/v)) for 1 hour at RT. After a further three washes with PBS, cells were incubated with primary antibody (please refer to **Table 2.4.**) for 2 hrs at RT. Secondary antibodies, raised in donkey and conjugated with either AF488 or AF555 (Invitrogen, UK), were then added after three washes in PBS, and cells were incubated at RT in the dark for 1 hour. After this, the cells were washed again, and mounted using Prolong Gold Anti-Fade (Invitrogen, UK) containing 4'6-diamidino-2-phenylindole (DAPI), cover-slipped and left overnight at RT in the dark to cure. Slides were examined the following day and images captured using an upright fluorescence microscope (Zeiss Axioscop 2) with an attached camera (Olympus C-35DA-2).

2.19. Data handling and statistical analysis

All data presented are the mean \pm standard errors of the mean (SEM) from triplicates (unless otherwise stated) of one experiment, and are representative of experiments performed on three independent occasions. Statistical comparison was performed between two treatment groups by student's t-test, and between multiple treatment groups by ANOVA (one-way

or two-way, as indicated in the relevant figure legend) with Tukey post hoc testing. A p value of less than 0.05 was considered significant. Whilst it is not uncommon for data to be presented in this manner, I acknowledge that a more valid approach would be to perform multivariate statistical analysis on replicates from all independent experiments. To demonstrate my understanding of this, data in Figure 3.16 is presented in this manner.

CHAPTER 3: RESULTS

Overview

The experimental results of this thesis have been divided into four sections. The first section addresses the cellular immunolocalisation of TSLP and TSLPR in human fibrotic lung samples. The second section examines the regulation of TSLP expression by human alveolar epithelial cells and lung fibroblasts. The third section evaluates the effect of TSLP on human lung fibroblasts. The fourth section address the immunolocalisation of TSLP and TSLPR in murine lungs following bleomycin-induced lung injury and further examines the role of TSLP in dendritic cell (DC) activation and the development of a Type-2 (T-2) immune response in this non-allergen driven model of lung injury. This section also investigates the role of TSLP in the subsequent development of lung fibrosis in this model.

3.1. The cellular immunolocalisation of TSLP and TSLPR in human lung fibrosis

3.1.1. Introduction

The expression of TSLP protein has been examined in a number of different conditions, all characterised by increased expression of T-2 cytokines (Taylor *et al.*, 2009; Ying *et al.*, 2005; Yoo *et al.*, 2005). In the lung, atopic asthma represents the archetypal T-2 inflammatory condition in which TSLP expression has been most extensively examined, both in terms of animal models and human disease, identifying bronchial epithelial cells and mast cells as potentially important sources of this cytokine. However, these cell types are not felt to significantly contribute to the pathogenesis of IPF, in contrast to alveolar epithelial cells and lung fibroblasts, as described in **Section 1.3.2.**

Lung fibroblasts represent the key effector cells responsible for the excessive deposition of extracellular matrix proteins which underpins the parenchymal fibrosis of fibrotic lung disease (Scotton *et al.*, 2007) and the airway remodelling characteristic of atopic asthma (Homer *et al.*, 2005). However, their close proximity to the epithelium means that they are also in a unique position to relay signals of epithelial injury to immune cells, highlighting a potential duality of function: ECM deposition and the direction of immune cell trafficking.

Despite airway remodelling being a characteristic feature of chronic asthma (Homer *et al.*, 2005), characterised by subepithelial fibrosis, no immunolocalisation of TSLP to fibroblasts has been reported in this condition. Moreover, to the best of my knowledge, the immunolocalisation of TSLP in IPF, a non-allergen driven lung disease also characterised by the increased expression of T-2 cytokines, has not been evaluated.

3.1.2. Immunohistochemical localisation of TSLP and TSLPR in human fibrotic lung

In order to determine the cellular localisation of TSLP and TSLPR in normal and fibrotic human lung, fibrotic and non-fibrotic lung sections were examined by immunohistochemistry. Lung biopsy specimens were obtained from 12 patients with IPF (obtained at diagnostic surgical lung biopsy) and 3 control patients (obtained from uninvolved tissue during cancer resection surgery). All biopsies used in this study were classified using the diagnostic criteria of the American Thoracic Society/European Respiratory Society Consensus (American Thoracic Society/European Respiratory Society International multidisciplinary consensus classification of the idiopathic interstitial pneumonias, 2002) demonstrating a pattern of Usual Interstitial Pneumonia (UIP).

Initial studies focused upon determining the optimal antigen unmasking technique suitable for detection of both TSLP and TSLPR, as described in **Materials and Methods**. Following appropriate antigen unmasking (**Section 2.11.2**), optimization of the concentration of TSLP detecting antibody was established on lung tissue sections. A dilution series was constructed and lung tissue sections were incubated with a biotinylated polyclonal sheep anti-TSLP antibody overnight at 4°C at varying concentrations. Positive staining for TSLP was detected by incubating sections with an optimal concentration of biotinylated antibody (2 µg/ml), followed by a streptavidin/peroxidase complex and a solution of 3,3'- diaminobenzidine (DAB), which generates a brown reaction product.

For optimization of the TSLPR detecting antibody, a similar protocol was observed. However, as the primary antibody was a non-biotinylated polyclonal rabbit anti-TSLP antibody, an additional incubation with a biotinylated goat anti-rabbit secondary antibody, prior to incubation with the streptavidin/peroxidase complex, was required. Positive staining for TSLPR was detected with an optimal concentration of 5 µg/ml.

TSLP and TSLPR immunostaining was considered to reflect a specific signal as there was no discernible staining observed on serial control fibrotic tissue sections incubated with isotype specific, non-immune primary antibodies.

Figure 3.1. shows the immunohistochemical localisation of TSLP in human fibrotic and non-fibrotic control lung samples. Tissue sections from control lungs showed weak positive staining, which was localised to occasional alveolar epithelial cells, and stronger staining associated to macrophages. However, tissue sections from fibrotic lung tissue showed widespread prominent staining which was predominantly associated with hyperplastic type II alveolar epithelial cells, macrophages and fibroblasts within fibrotic foci. Strong immunoreactivity for TSLP was also observed on fibroblasts in areas of established fibrosis. The degree of TSLP immunoreactivity localising to macrophages was similar in both non-fibrotic control lung and human IPF. The intensity of TSLP immunoreactivity for alveolar epithelial cells, fibroblasts and macrophages, from each of all 12 patients with IPF and the 3 control samples were graded from absent to very strong, and are summarised in **Table 3.1**. These data demonstrate that the pattern of immunoreactivity described above was a consistent finding.

Figure 3.2. shows the immunohistochemical localisation of TSLPR in human fibrotic and non-fibrotic control lung samples. Tissue sections from control lungs demonstrated strong staining for bronchial epithelial cells and macrophages. Occasional staining was observed for alveolar epithelial cells, though the intensity for this cell type was variable. In contrast. sections from fibrotic lungs demonstrated intense staining for alveolar epithelial cells, fibroblasts and macrophages. In addition, strong immunoreactivity was observed for mononuclear cells with the morphological characteristics of lymphocytes. Such cells were generally organised in structures resembling lymphoid follicles, as has been described previously in IPF (Marchal-Somme *et al.*, 2006). Moreover, cells with the morphological characteristics of DC were also observed to demonstrate staining for TSLPR. These cells were generally intercalated with mononuclear cells within the follicular structures described above.

As described in **Section 1.4.3.**, expression of TSLP is upregulated by a number of mediators, including TNF- α and IL-1 β . Such cytokines may be regarded as early wave “alarm” cytokines, reflecting cellular injury in response to a variety of stimuli. To evaluate the structural relationship of cells immunoreactive for TNF- α , TSLP and DC, a major cellular target for TSLP, serial sections of human IPF lung were stained

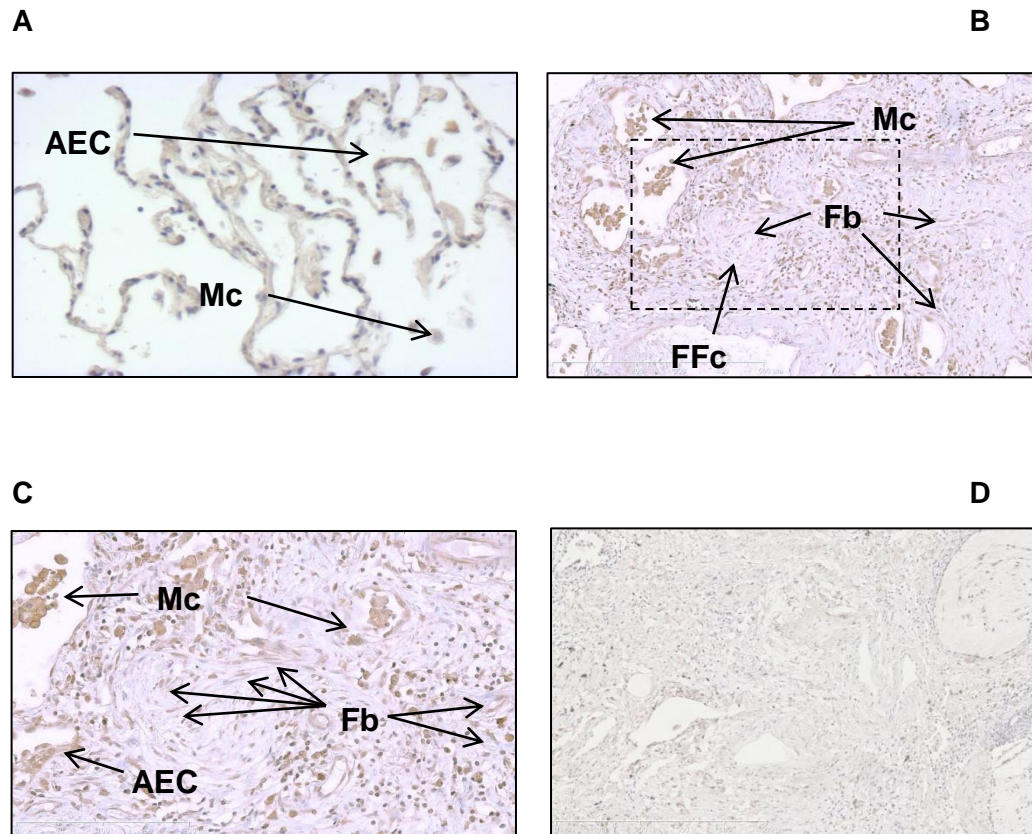


Figure 3.1.

TSLP immunostaining in Idiopathic pulmonary fibrosis (IPF) identifies epithelial cells, fibroblasts and macrophages as major cell types displaying strong immunoreactivity.

Shown is immunostaining for TSLP (**A-C**) in normal non-fibrotic control lung (**A**) and IPF lung (**B and C**). In control lung (**A**), TSLP immunoreactivity is observed for macrophages (Mc); weak TSLP staining is also observed for alveolar epithelial cells (AEC). In contrast, in IPF lung (**B,C**), strong immunoreactivity for TSLP is observed for macrophages, cells with the morphology of fibroblasts (Fb) and alveolar epithelial cells. Fibroblasts in the IPF lung are located with a fibrotic focus (FFc, dotted area, seen at higher magnification in panel (**C**)), the characteristic histological feature of IPF. Serial IPF tissue section stained with isotype-specific non-immune primary antibody (**D**) shows no discernible staining. Original magnifications x20 **A, C**; x10 **B, D**. n=12 for IPF lungs and n=3 for normal lung. Sections shown are representative of all patients observed.

	Alveolar epithelial cell	Fibroblasts within fibrotic foci	Fibroblasts within areas of established fibrosis	Macrophages
1	++	+	+/-	+
2	+	++	+	+
3	++	+	+	+
4	++	+	+	++
5	+	++	+	++
6	-/+	+	++	++
7	+++	++	++	+++
8	++	++	+	+
9	+++	++	++	++
10	++	+	+	+++
11	+	+/-	+	++
12	++	+	++	++
A	-	-	n/a	+++
B	+/-	-	n/a	+
C	-	-	n/a	++

Table 3.1.

The immunolocalisation of TSLP in human IPF lung (samples 1-12) compared to non-fibrotic control lung (samples A-C). Data presented as – (absent) to very strong (+++) immunostaining for TSLP; n=12, IPF; n=3, control.

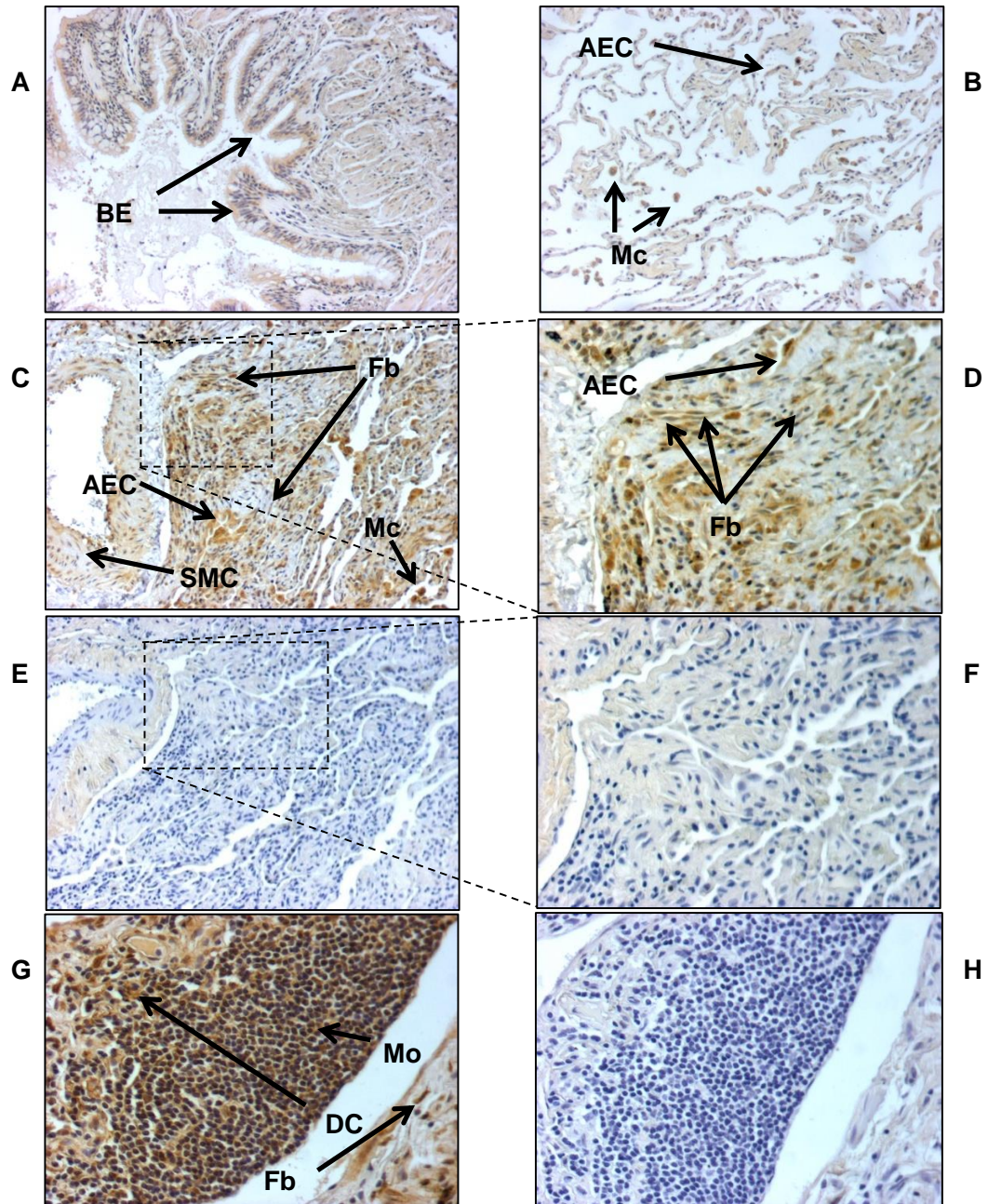


Figure 3.2.

TSLPR immunostaining in Idiopathic pulmonary fibrosis (IPF) identifies epithelial cells, fibroblasts and immune cells as major cell types displaying strong immunoreactivity.

Shown is immunostaining for TSLPR (**A-D, G**) in normal non-fibrotic control lung (**A and B**) and IPF lung (**C and D**). In control lung (**A and B**), TSLP immunoreactivity is observed for bronchial epithelium (BE) and macrophages (Mc). Weak TSLPR staining is also observed for alveolar epithelial cells (AEC). In contrast, in IPF lung (**C and D**), strong immunoreactivity for TSLP is observed for macrophages, cells with the morphology of fibroblasts (Fb) and alveolar epithelial cells, as well as smooth muscle cells (SMC). TSLP immunoreactivity is also observed on cells with the shape of dendritic cells (DC), as well as on mononuclear cells (Mo) within lymphoid aggregates (G). Serial IPF tissue section stained with isotype-specific non-immune primary antibody (**E, F and H**) shows no discernible staining. Original magnifications x10 **A, B, C, E**; x20 **D, F-H**. n=3 for IPF lungs and n=3 for normal lung. Sections shown are representative of all patients observed.

for the aforementioned ligands and for CD11c, a DC marker. **Figure 3.3.** demonstrates the immunolocalisation of TNF- α , TSLP and CD11c on serial human IPF sections. Strong immunoreactivity for TNF- α was observed for hyperplastic alveolar epithelium. This positive staining was observed to extend into the adjacent underlying extracellular matrix surrounding fibroblasts. Strong TSLP immunoreactivity was again observed for cells with the morphological characteristics of fibroblasts underlying hyperplastic epithelium, as well as for macrophages. In addition, mononuclear cells organised within lymphoid aggregates displayed positive TSLP staining. Finally, cells with the morphology of dendritic cells were observed to be strongly positive for CD11c. These cells were predominantly located within lymphoid follicles, though alveolar macrophages, which are also recognised to express CD11c, stained positive also, and were observed in alveolar spaces.

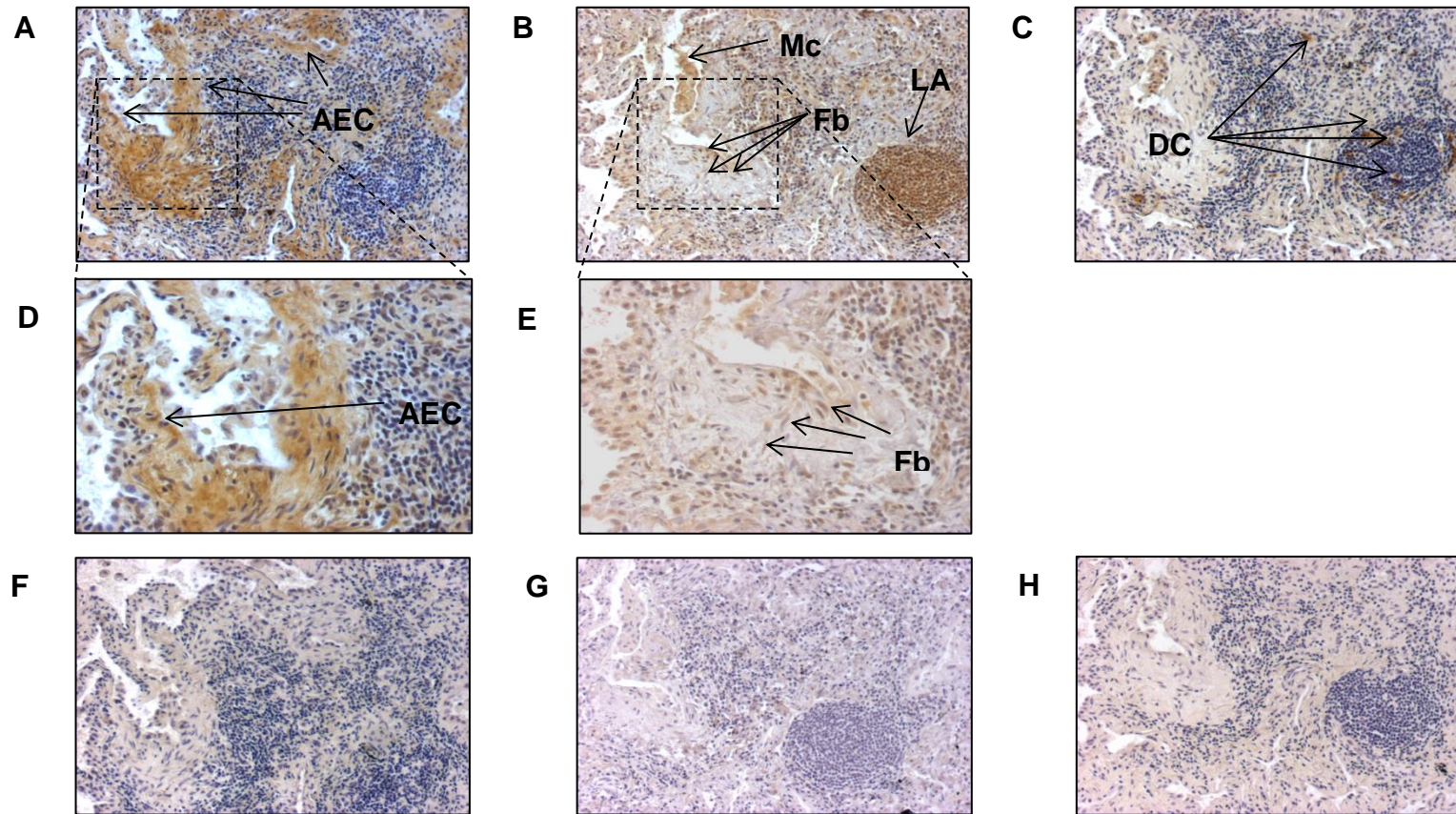


Figure 3.3. Serial immunodetection of TNF- α , TSLP and CD11c demonstrates the structural proximity of TNF- α expressing epithelial cells, TSLP-expressing fibroblasts and dendritic cells in IPF. Strong immunoreactivity for TNF- α is observed for hyperplastic epithelial cells (AEC) and for fibroblast-associated matrix (**A, D**). Cells with morphology of fibroblasts (Fb) in close proximity display strong immunoreactivity for TSLP, in addition to macrophages (Mc) and mononuclear cells within lymphoid aggregates (LA) (**B, E**). Strongly positive CD11c cells, with the characteristic morphology of dendritic cells (DC) are observed within lymphoid aggregates in close proximity to TSLP immunoreactive cells (**C**). No discernible staining was observed for TNF- α , TSLP and CD11c in serial sections stained with isotype-specific non-immune primary antibodies (**F-H**). Original magnification x10 (**A-C; F-H**); x20 (**D, E**).

3.1.3. Summary

The results described in this section, examining the immunolocalisation and potential cellular sources of TSLP, as well as that of its receptor show that:

- TSLP and TSLPR immunoreactivity is greater in fibrotic human lung tissue compared to non-fibrotic lung tissue;
- in fibrotic human lung tissue, TSLP immunolocalises to alveolar type II epithelial cells (AECII), fibroblasts and macrophages, and TSLPR immunolocalises to AECII, fibroblasts, mononuclear cells and dendritic cells;
- in fibrotic human lung tissue, alveolar epithelial cells, fibroblasts and dendritic cells, strongly immunoreactive for TNF- α , TSLP and CD11c respectively, are observed in close structural proximity;

In summary, the results presented in this section show that TSLP and TSLPR immunolocalises to epithelial cells, fibroblasts and macrophages in human fibrotic lungs, with greater immunoreactivity observed in fibrotic compared to non-fibrotic lungs. In addition, TSLPR immunolocalises to cells with the morphology of dendritic cells in human fibrotic lung disease. These data suggest that expression of TSLP is upregulated in IPF, identifying for the first time alveolar epithelial cells and fibroblasts as possible cellular sources of this cytokine in human lung fibrosis. Moreover, they identify lung fibroblasts as a novel cell type expressing TSLPR in IPF, suggesting that human lung fibroblasts may represent a cellular target for TSLP in IPF.

3.2. The role of TNF- α in inducing TSLP expression in primary human lung fibroblasts and lung epithelial cells

3.2.1. Introduction

TSLP has traditionally been regarded as an epithelium-derived cytokine (Liu *et al.*, 2007b), and its expression in the lung has been examined predominantly in airway epithelial cells (Allakhverdi *et al.*, 2007a; Lee *et al.*, 2007a), reflecting its perceived importance in the pathogenesis of atopic asthma. However, although bronchiolarization of the alveolar epithelium is a recognised feature of IPF (Kawanami *et al.*, 1982), the alveolar, rather than airway, epithelium is felt to be the primary site of disease initiation in this condition, with the predominant downstream effector cell being the (myo)fibroblast. In light of the strong immunoreactivity observed for TSLP in human IPF on hyperplastic alveolar epithelial cells and fibroblasts, I was keen to determine which of these cell types represented the likely source of this cytokine in this disease. For these studies, I examined the effect of TNF- α on TSLP expression by lung epithelial cells and fibroblasts *in vitro*, as this master cytokine has previously been demonstrated to be a potent inducer of TSLP expression in other cell types (Lee *et al.*, 2007a). Moreover, TNF- α expression is known to be upregulated in IPF (Ziegenhagen *et al.*, 1998), and the present studies have demonstrated the close structural proximity of injured epithelium to fibroblasts within fibrotic foci, immunoreactive for TNF- α and TSLP respectively (Section 3.1.2.).

3.2.2. Effect of TNF- α on TSLP protein production by lung epithelial cells

In order to explore whether the alveolar epithelium represented a potential cellular source of TSLP in IPF, the effect of TNF- α on freshly isolated primary type II alveolar epithelial cells from donor lung and A549 cells was examined. Cells were exposed to TNF- α (10 ng/ml) and TSLP protein levels in culture supernatants were assessed by ELISA. TSLP protein in conditioned media (CM) was not detectable from any of these cell types at any of the time points chosen (0, 1, 2, 4, 8, 12 and 24 hours) following incubation with TNF- α . Data not shown.

3.2.3. Effect of TNF- α on TSLP protein production by primary human lung fibroblasts (pHLFs)

In addition to hyperplastic epithelial cells, fibroblasts, within fibrotic foci, demonstrated strong immunoreactivity for TSLP in IPF lung sections. In order to

explore the fibroblast as potential source of TSLP in this condition, baseline production and expression following exposure to TNF- α was examined using primary human lung fibroblasts (pHLFs) grown from explant cultures in our laboratory (please see **Materials and Methods**). pHLFs were exposed to varying concentrations of TNF- α , and TSLP protein levels in CM were assessed by ELISA.

Initial studies (**Figure 3.4. A-B**) showed that baseline production of TSLP by pHLFs was low (between 25 and 30 pg/ml) and did not change over 24 hours (**Panel A**). In contrast, TNF- α (10 ng/ml) promoted TSLP protein release in a time-dependent manner. The sharpest increase in TSLP protein release was observed between 4 and 8 hours. Thereafter, the increase in TSLP protein release was maintained though blunted, although no plateau in this response was observed. Extending this time course to 72 hours (**Panel B**) confirmed that TSLP protein release in untreated cells remained constant and low (~50 pg/ml). However, in cells exposed to TNF- α , TSLP protein release continued beyond 24 hours though the increase between 24 and 72 hours was less pronounced than that observed between 0 and 24 hours. These data therefore suggest that pHLFs remain responsive to TNF- α , with respect to TSLP protein release, up to 72 hours after initial exposure.

Having demonstrated that TNF- α promotes TSLP protein in a time-dependent manner, I was keen to investigate the kinetics of this interaction further by examining the concentration-response profile of this effect. As can be seen from **Figure 3.4. (A)**, TNF- α induces a significant increase in TSLP protein release after a 4 hours incubation period, though a statistically more significant response was observed from 6 hours. Hence, this time-point was chosen to examine the concentration-dependent effect of TNF- α on TSLP protein release by pHLFs. **Figure 3.5.** shows that TNF- α promotes TSLP protein release in a concentration-dependent manner between 0.03 and 0.3 ng/ml. Concentrations of between 0.3 and 30 ng/ml did not induce any further increase in TSLP protein release. In contrast, concentrations of 100 ng/ml did promote further TSLP protein release. However, this concentration of TNF- α is of doubtful physiological or pathological relevance, and so for future studies, a concentration of 10 ng/ml was used

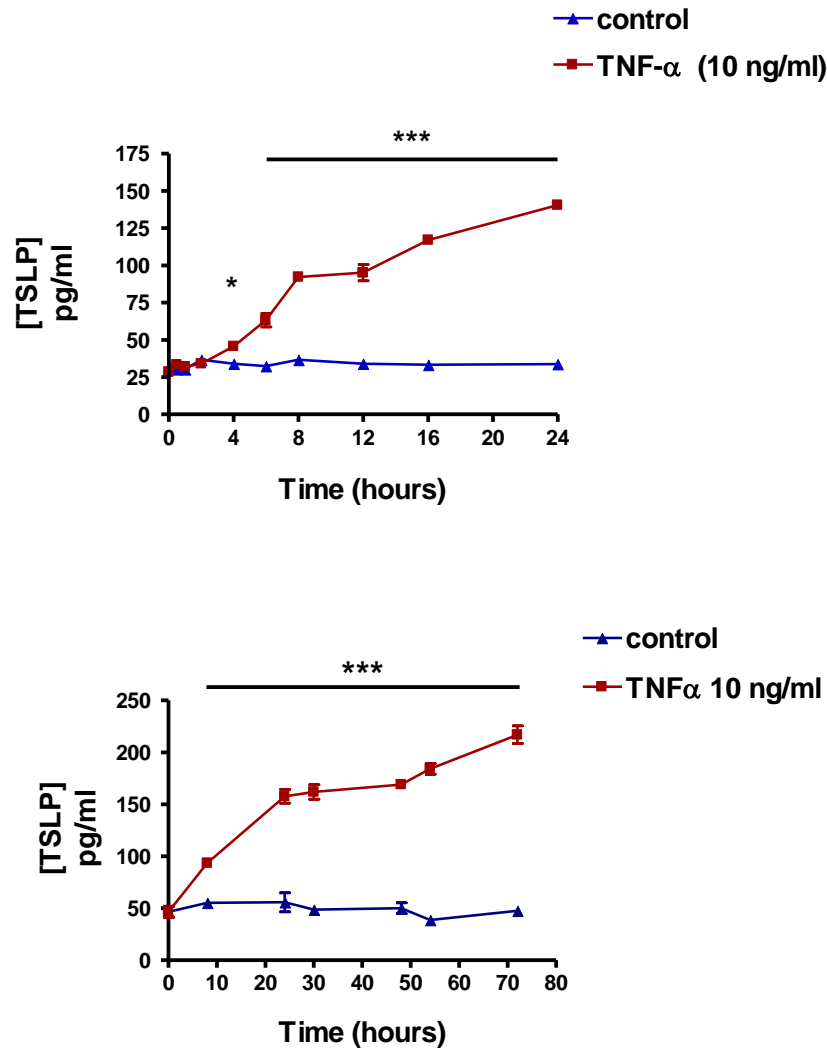


Figure 3.4.

TNF- α stimulates TSLP protein release by primary human lung fibroblasts (pHLFs) in a time-dependent manner.

Figure shows time course data for the effect of TNF- α on pHLF TSLP protein release into conditioned media performed over 24 hours (**Panel A**) or 72 hours (**Panel B**). pHLFs were exposed to TNF- α (10 ng/ml) for varying durations (**A**: 0-24 hours; **B**: 0-72 hours). Cell culture supernatants were analysed for TSLP protein release by ELISA. The amount of secreted TSLP is expressed as pg/ml, and each value represents the mean \pm SEM, from triplicates. * $p < 0.05$; *** $p < 0.001$, comparison with time-point matched media control; two-way ANOVA.

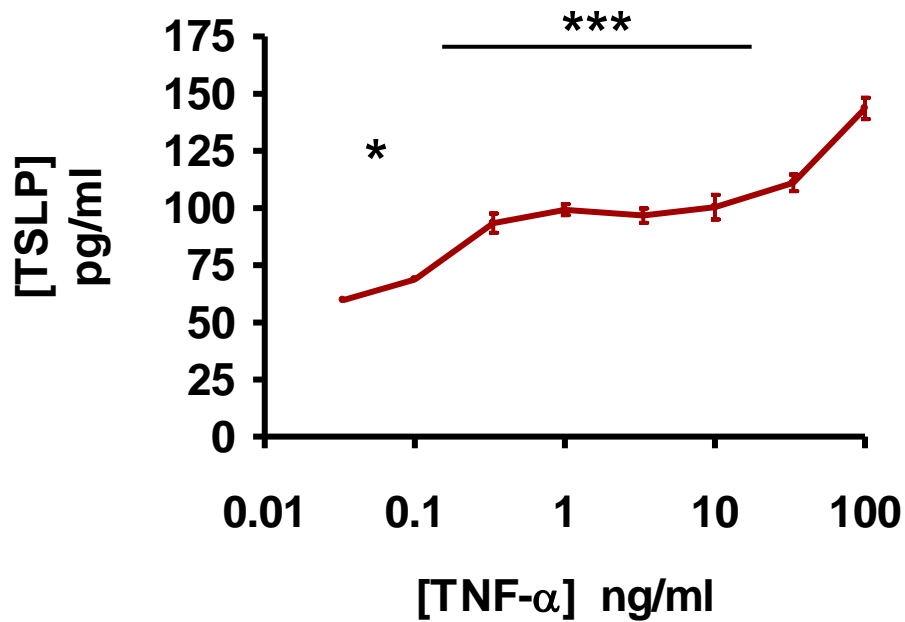


Figure 3.5.

TNF- α stimulates pHLF TSLP protein release in a concentration-dependent manner.

Figure shows concentration-response data for the effect of TNF- α on pHLF TSLP protein release into conditioned media. pHLFs were exposed to serum-free control medium or TNF- α (0.03 – 100 ng/ml) for 6 hours. Conditioned media were analysed for TSLP protein release by ELISA. The amount of secreted TSLP is expressed as pg/ml, and each value represents the mean \pm SEM from triplicates. * $p < 0.05$; *** $p < 0.001$, comparison with unstimulated cells; one-way ANOVA.

3.2.4. Effect of TNF- α on TSLP mRNA levels in pHLFs

The data obtained so far strongly indicate that TNF- α induces TSLP protein release from pHLFs in a concentration- and time-dependent manner. To determine if TNF- α exerts these effects by influencing *Tslp* gene expression, the effect of TNF- α on TSLP mRNA levels was assessed by qRT-PCR.

pHLFs were found to express TSLP mRNA transcripts at baseline (**Figure 3.6.**). Exposure of cells to TNF- α (10 ng/ml) resulted in a significant increase in TSLP mRNA levels within 2 hours, with a maximal increase (over 50-fold relative to control) at 4 hours. TSLP mRNA levels diminished thereafter and returned to baseline by 24 hours.

Two splice variants for human TSLP have previously been reported (Harada *et al.*, 2009). The splice variants use different initiation methionine codons for protein translation. Previous expression studies (Kato *et al.*, 2007; Lee *et al.*, 2007a) had not examined mRNA expression of the two splicing variants separately. The relative frequency of these splice variants has, to date, not been determined. To determine which splice variant of TSLP was induced by TNF- α in pHLFs, the effect of TNF- α on TSLP short and long splice transcript levels was assessed by qRT-PCR. Since the maximal fold induction in TSLP mRNA levels, relative to control, was observed at 4 hours, this time point was chosen for these studies.

Figure 3.7. (A and B) shows that both short and long splice variants of TSLP transcripts were detected at baseline and that TNF- α (10 ng/ml) significantly increased the expression of the long TSLP splice variant only.

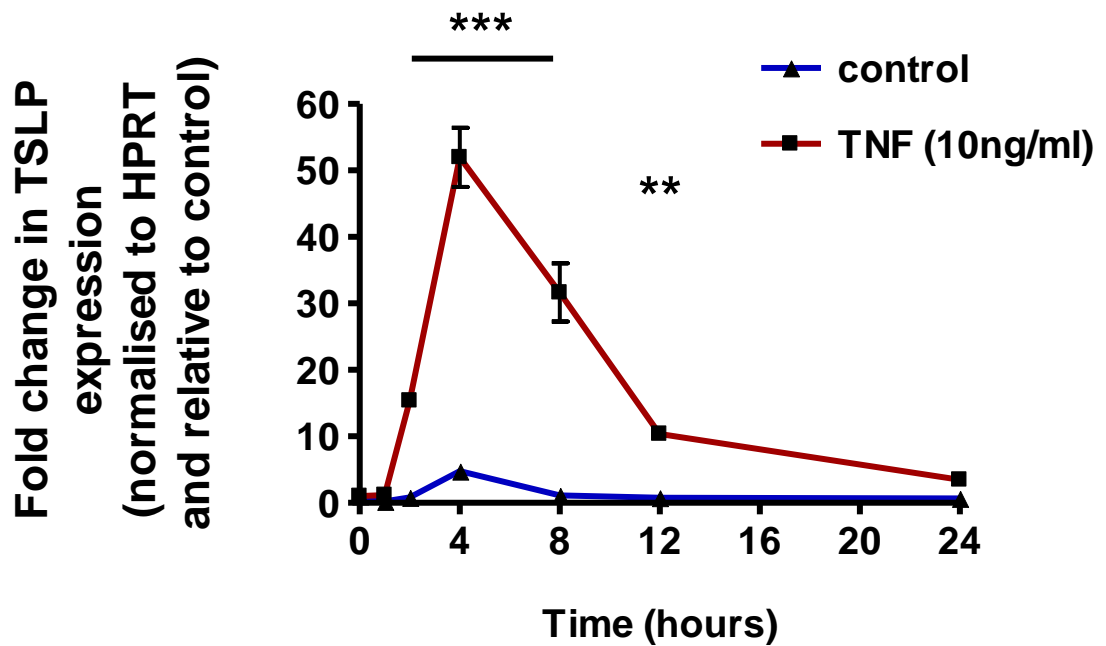


Figure 3.6.

TNF- α increases TSLP mRNA levels in pHLFs.

Figure shows time-course data for the effect of TNF- α on pHLF TSLP mRNA levels. pHLFs were exposed to serum-free control medium or TNF- α (10 ng/ml) for varying durations from 0-24 hours. TSLP mRNA levels at each time point were assessed by qRT-PCR. Data are expressed as fold-change relative to time zero, normalised to the house-keeping gene hypoxanthinephosphoribosyltransferase (HPRT) mRNA levels (mean \pm SEM from triplicates); ** p <0.01; *** p <0.001, comparison with time-matched media controls; two-way ANOVA.

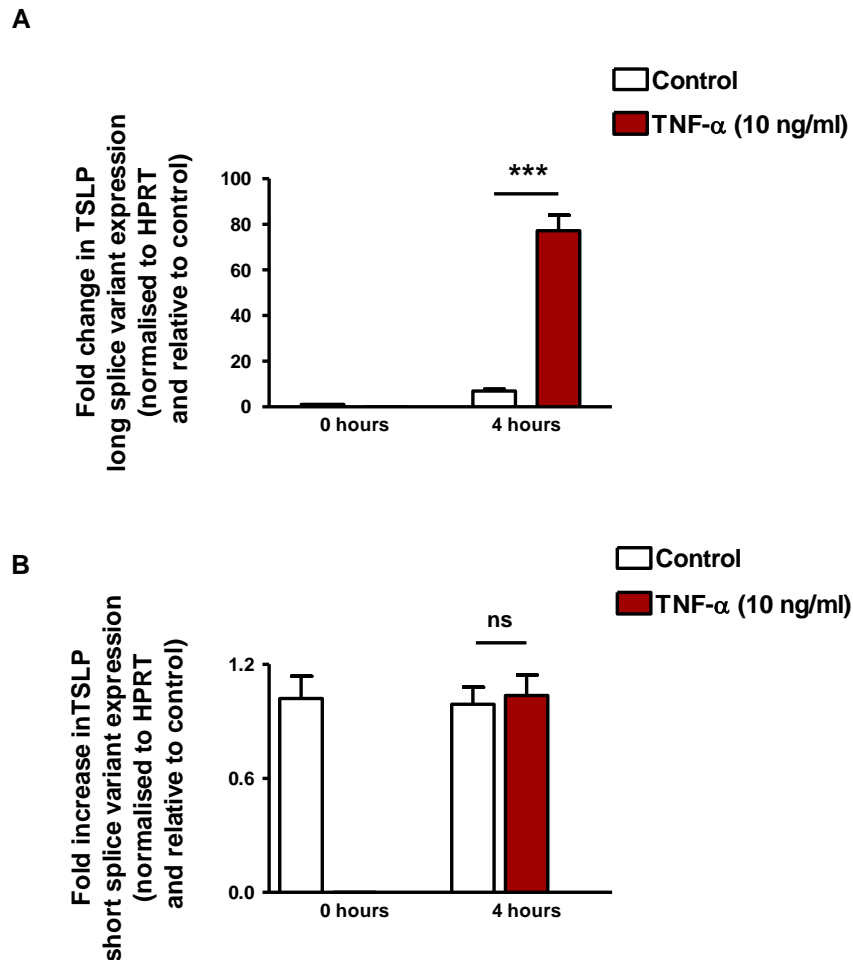


Figure 3.7.

TNF- α only increases TSLP long splice variant mRNA levels in pHLFs.

Panel shows the effect of TNF- α on pHLF TSLP mRNA levels (**A**- long splice variant; **B**- short splice variant). pHLFs were exposed to serum-free control medium or TNF- α (10 ng/ml) for 4 hours. TSLP mRNA levels were assessed by qRT-PCR. Data are expressed as fold change relative to time zero for each time point (mean \pm SEM from triplicates) following normalization to HPRT mRNA levels. *** $p < 0.001$, comparison with time-matched medium control; one-way ANOVA.

3.2.5. Effect of NF κ B inhibition on TNF- α -induced TSLP protein production in pHLFs

Binding of TNF- α to its major receptor, TNFRI, ultimately results in the activation of two major transcription factors, NF κ B and AP-1. Previous studies have suggested the importance of both these factors in mediating TSLP expression in epithelial cells, as discussed in **Section 1.4.3**. Studies were therefore performed to examine the role of NF κ B in TNF- α induced TSLP expression in pHLFs using a highly specific inhibitor of IKK-2, SC-514, that has previously been shown to inhibit NF κ B-dependent gene expression in fibroblasts (Kishore *et al.*, 2003; Sullivan *et al.*, 2009).

3.2.5.1. Effect of TNF- α on NF κ B signalling in pHLFs

As discussed in **Section 1.4.3.3.**, liberation of NF κ B from its basal ternary complex with I κ B proteins is achieved following phosphorylation, ubiquitination and degradation of I κ B proteins, allowing the translocation of NF κ B homodimers into the nucleus where they can bind to regulatory components of target genes. Loss of I κ B signal on Western blotting therefore represents liberation of NF κ B dimers for transcriptional regulation and initiation of this signalling pathway. Initial studies examined the effect of TNF- α on NF κ B signalling. pHLFs were exposed to TNF- α (10 ng/ml) or control medium for varying durations, ranging from 0 to 60 minutes. Cell lysates were analysed by Western blotting and probed with an anti-I κ B antibody. As can be seen from **Figure 3.8. (A)**, marked loss of the I κ B signal, suggestive of NF κ B signalling, was observed within 5 minutes of exposure of pHLFs to TNF- α . Recovery of the I κ B signal was observed at 60 minutes suggesting that NF κ B signalling had returned to baseline.

The effect of SC-514 on the NF κ B pathway was then examined. Cells, pre-incubated with increasing concentrations of SC-514 for 30 minutes, were exposed to TNF- α (10 ng/ml) for 15 minutes. Cell lysates were again examined by Western blotting as above. **Figure 3.8. (B)** shows that SC-514 inhibits TNF- α -induced loss of the I κ B signal in a concentration dependent manner, confirming that this molecule inhibits the NF κ B pathway. Marked inhibition of NF κ B signalling was observed for SC-514 concentrations of 1 μ M (-log 6 M) upwards.

3.2.5.2. *Effect of SC-514 on TNF- α induced TSLP protein production in pHLFs*

Having demonstrated that SC-514 is an effective inhibitor of I κ B degradation at concentrations greater than 1 μ M, I then examined the effect of SC-514 on TNF- α -induced TSLP protein release. pHLFs were incubated with increasing concentrations of SC-514 (0 – 30 μ M) for 30 minutes, prior to exposure to TNF- α (10 ng/ml) or control medium control medium for 6 hours. Conditioned media were then analysed for TSLP protein release by ELISA. **Figure 3.9.** shows that SC-514, up to concentrations of 3 μ M (-log 5.5 M), had no effect on TNF- α induced TSLP protein release. Significant inhibition of TSLP protein release was only observed at SC-514 concentrations of 10 μ M (-log 5 M) and above. Given that SC-514 already inhibits TNF- α induced degradation of I κ B at concentrations around 1 μ M, it is likely that the observed inhibition of TSLP protein release observed at higher concentrations reflect off-target effects of this inhibitor. These data therefore suggest that NF κ B does not play a significant role in mediating TNF- α -induced TSLP protein release in pHLFs.

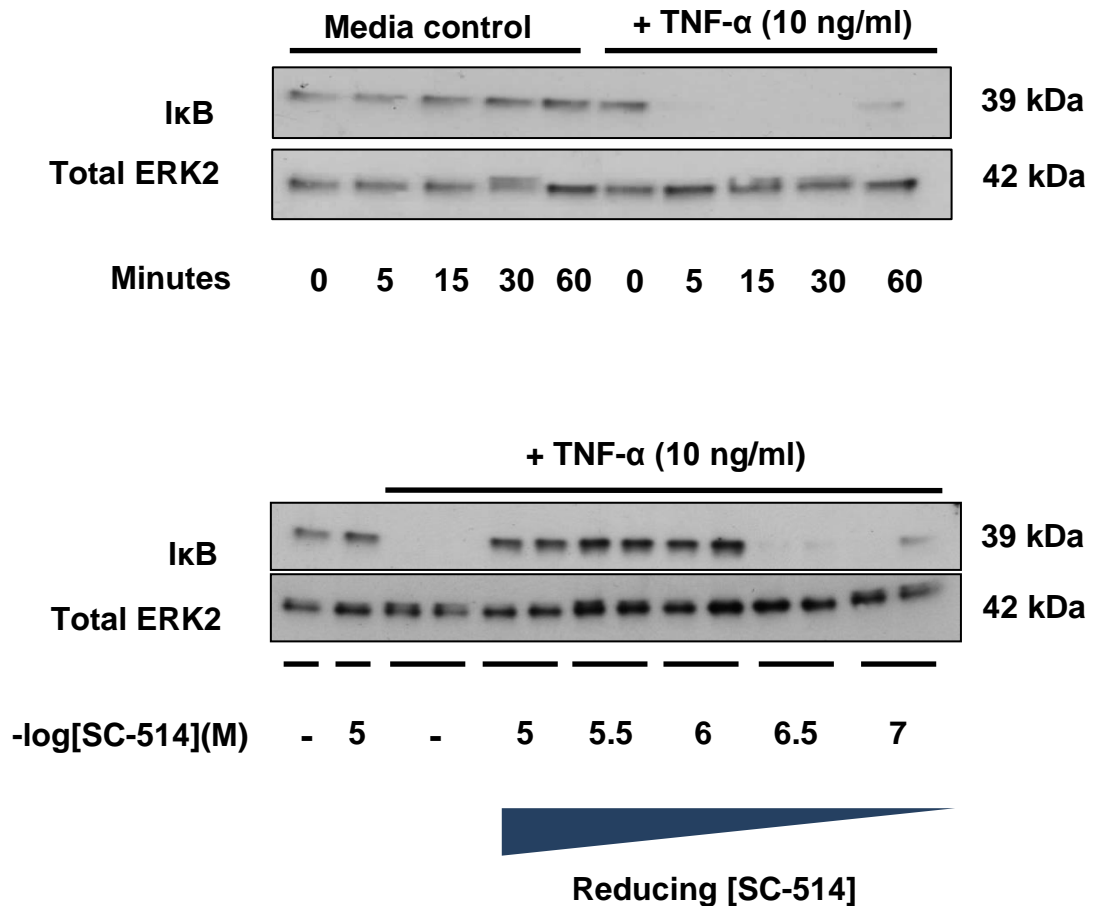


Figure 3.8.

TNF-α induces activation of NFκB in a time-dependent manner and this effect is inhibited by SC-514 in pHLFs.

Panel (A) shows that TNF-α induces the activation of NFκB signaling in a time-dependent manner. pHLFs were exposed to control medium or TNF-α (10 ng/ml) for the indicated time periods. Activation of NFκB signaling was assessed by Western blotting using an anti-IκB antibody to detect IκB protein levels as an index of NFκB signaling (**upper panel, A**). Protein loading was confirmed by blotting with an anti-total ERK2 antibody (**lower panel, A**). **Panel (B)** shows the effect of SC-514 on TNF-α-induced NFκB activation. Cells were pre-incubated with varying concentrations of SC-514 for 30 minutes prior to exposure to control medium or TNF-α (10 ng/ml) for 15 minutes. IκB levels and protein loading was assessed as above. Data are representative of three separate experiments performed.

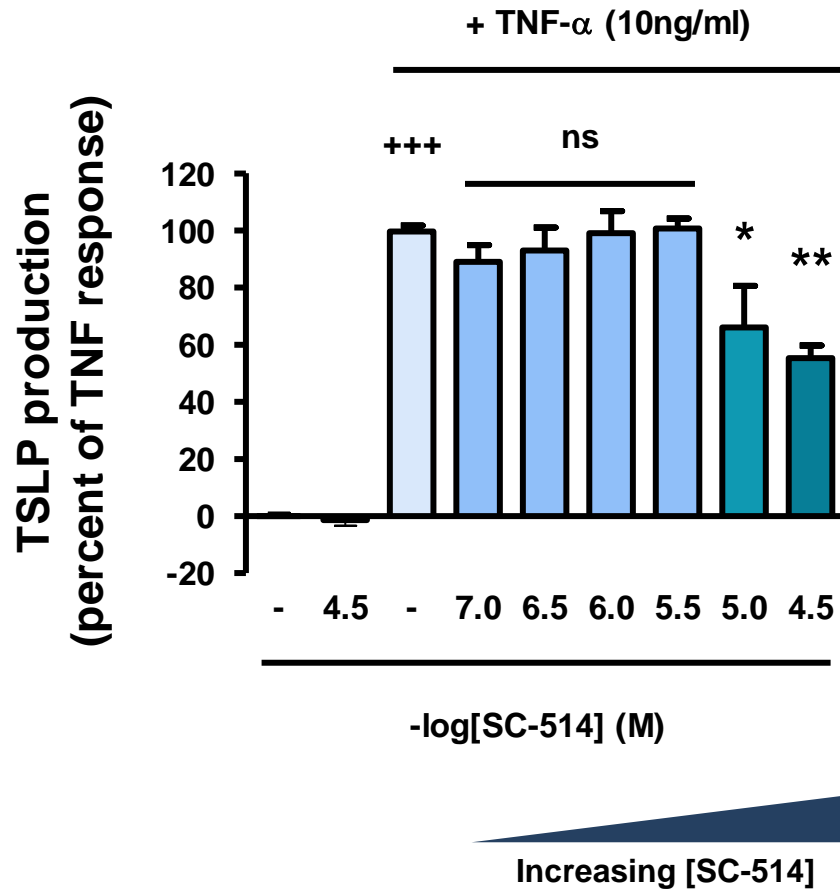


Figure 3.9.

TNF- α -induced TSLP protein release in pHLFs is not attenuated in a concentration-dependent manner by the NF κ B inhibitor, SC-514.

Figure shows the effect of the specific NF κ B inhibitor, SC-514, on TSLP protein release by pHLFs in response to TNF- α stimulation. Data are presented as a percentage of the maximal response obtained with TNF- α and drug vehicle alone (0.1% DMSO in DMEM). Cells were treated with increasing concentrations of SC-514 for 30 minutes prior to exposure to TNF- α (10 ng/ml) for 6 hours. Final concentrations of DMSO were kept constant for all experimental conditions. The first bar represents the TSLP response to control medium and drug vehicle alone. The second bar represents the highest concentration of SC-514 examined and shows that this compound has no effect on basal TSLP release. Negative log of the concentrations of SC-514 are presented. Data represent the mean \pm SEM, from triplicates. ns-non-significant; *p<0.05; **p<0.01, comparison with TNF alone; +++p<0.001, comparison with untreated cells; one-way ANOVA.

3.2.6. Effect of AP-1 inhibition on TNF- α -induced TSLP protein production in pHLFs

As discussed in **Section 1.4.3.2.**, AP-1 has been reported to play a role in mediating the expression of TSLP in epithelial cells (Harada *et al.*, 2009). Moreover, AP-1 is a major transcription factor involved in mediating many of the cellular effects of TNF- α in a wide variety of cells, including lung fibroblasts (Sullivan *et al.*, 2009). Therefore, the role of this transcription factor in mediating TNF- α induced TSLP expression in pHLFs was examined using an AP-1 inhibitor, curcumin, which has previously been demonstrated to inhibit TNF- α -induced AP-1 activity in lung fibroblasts (Sullivan *et al.*, 2009).

pHLFs were incubated with increasing concentrations of curcumin (0 – 30 μ M) prior to exposure to TNF- α (10 ng/ml) or control medium for 6 hours. Conditioned media was analysed for TSLP protein release by ELISA. **Figure 3.10.** shows that at concentrations of 3 μ M (-log 5.5 M) and above, significant inhibition was observed with abrogation of TSLP protein release observed at curcumin concentrations around 30 μ M (-log 4.5 M).

Although capable of an impressive inhibitory effect on TNF- α -induced TSLP protein release, curcumin has been reported to influence the activity of a number of signalling pathways, in addition to AP-1 (Plummer *et al.*, 1999), and so further studies were required before assigning a role for this factor in mediating this response.

3.2.7. Effect of MAP kinase inhibition on TNF- α -induced TSLP protein production in pHLFs

Differential phosphorylation of the components of AP-1, including c-Jun, is required to initiate AP-1 directed transcription of target genes, and this is mediated primarily by activated mitogen-activated protein kinases (MAPKs). TNF- α is known to exert many of its cellular effects via MAP kinase (p38, JNK and ERK1/2 kinases) pathways (Sullivan *et al.*, 2005; Ventura *et al.*, 2003). Moreover, previous studies have suggested that MAPK signalling may play a role in mediating TNF- α -induced TSLP expression in epithelial cells (Tu *et al.*, 2007; Zhang *et al.*, 2007). Therefore, the following series of experiments were initiated to examine the role of MAPKs in mediating TNF- α -induced TSLP protein release in pHLFs.

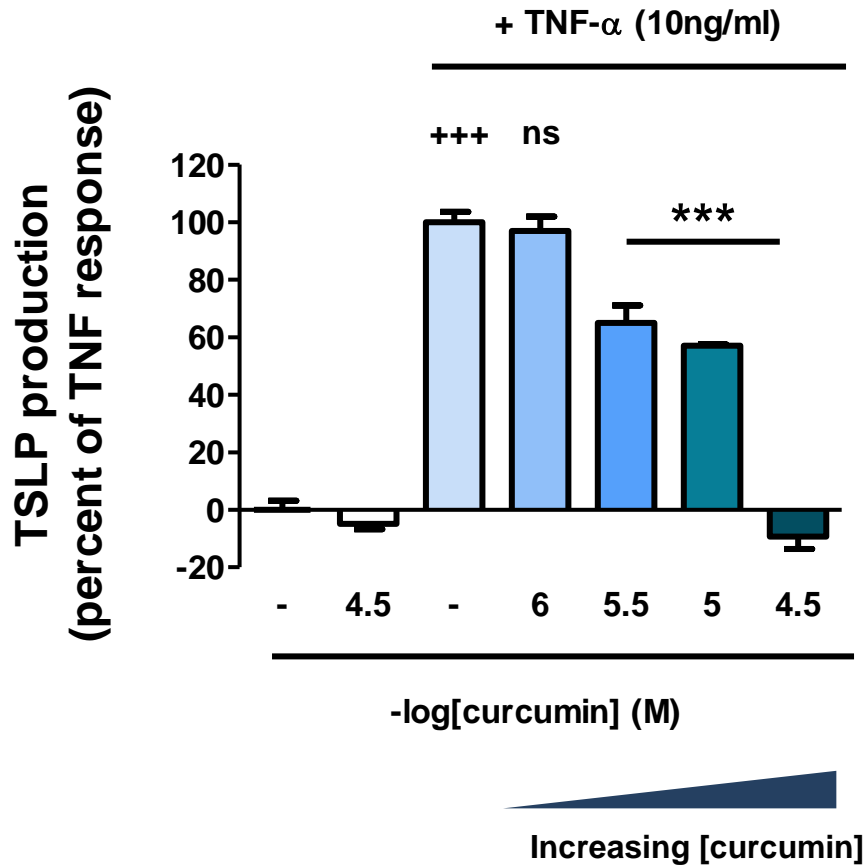


Figure 3.10.

TNF- α -induced TSLP protein release in pHLFs is attenuated in a concentration-dependent manner by the AP-1 inhibitor, curcumin.

Figure shows the effect of the specific AP-1 inhibitor, curcumin, on TSLP protein release by pHLFs in response to TNF- α stimulation. Data are presented as a percentage of the maximal response obtained with TNF- α and drug vehicle alone (0.1% DMSO in DMEM). Cells were treated with increasing concentrations of curcumin for 30 minutes prior to exposure to TNF- α (10 ng/ml) for 6 hours. Final concentrations of DMSO were kept constant for all experimental conditions. The first bar represents the TSLP response to control medium and drug vehicle alone. The second bar represents the highest concentration of curcumin examined and shows that this compound has no effect on basal TSLP release. Negative log of the concentrations of curcumin are presented. Data represent the mean \pm SEM from triplicates. ns-non significant; *** p <0.001, comparison with TNF- α alone; +++ p <0.001, comparison with untreated cells; one-way ANOVA.

3.2.7.1. *The effect of inhibition of p38 activity on TNF- α induced TSLP protein release in pHLFs*

The effect of inhibition of the p38 pathway on TNF- α induced TSLP protein release was examined using the specific inhibitor of p38 activity, SB203580. This compound has previously been demonstrated to inhibit p38 activity in fibroblasts (Reunanen *et al.*, 2002). In the first instance, TNF- α -induced activation of the p38 signalling pathway in pHLFs was examined. Cells were exposed to TNF- α (10 ng/ml) or control medium for varying durations (0 – 60 minutes). Activation of the p38 signalling pathway was examined by Western blotting using an anti-phospho heat shock protein (HSP) 27 antibody. HSP27 is a major downstream phosphorylation target of p38 MAP kinase and HSP27 phosphorylation serves as a surrogate marker for p38 activity (Xu *et al.*, 2006). **Figure 3.11. (A)** shows TNF- α induces the phosphorylation of HSP27 from 15 mins onwards, with return to baseline by 60 minutes.

The effect of SB203580 on p38 activity was then examined. Cells, pre-incubated with increasing concentrations of SB203580 (0 – 20 μ M) for 30 minutes, were exposed to TNF- α (10 ng/ml) for 15 minutes. Cell lysates were again examined by Western blotting as above. **Figure 3.11. (B)** shows that SB203580 inhibits TNF- α -induced phosphorylation of HSP27 in a concentration-dependent manner. Marked inhibition of HSP27 phosphorylation was observed for SB203580 concentrations of 2 μ M (-log 6.7 M) and above.

Having demonstrated that SB20358 is an effective inhibitor of p38 activity, I then examined its effect on TNF- α -induced TSLP protein release. pHLFs were incubated with increasing concentrations of SB20358 (0 – 20 μ M) for 30 minutes, prior to exposure to TNF- α (10 ng/ml) or control medium for 6 hours and TSLP protein release into conditioned media was analysed by ELISA. **Figure 3.12.** shows that SB20358, up to concentrations of 20 μ M (-log 7.7 M), had no effect on TNF- α induced or basal TSLP protein release. These data therefore suggest that p38 does not mediate TNF- α -induced TSLP expression in pHLFs.

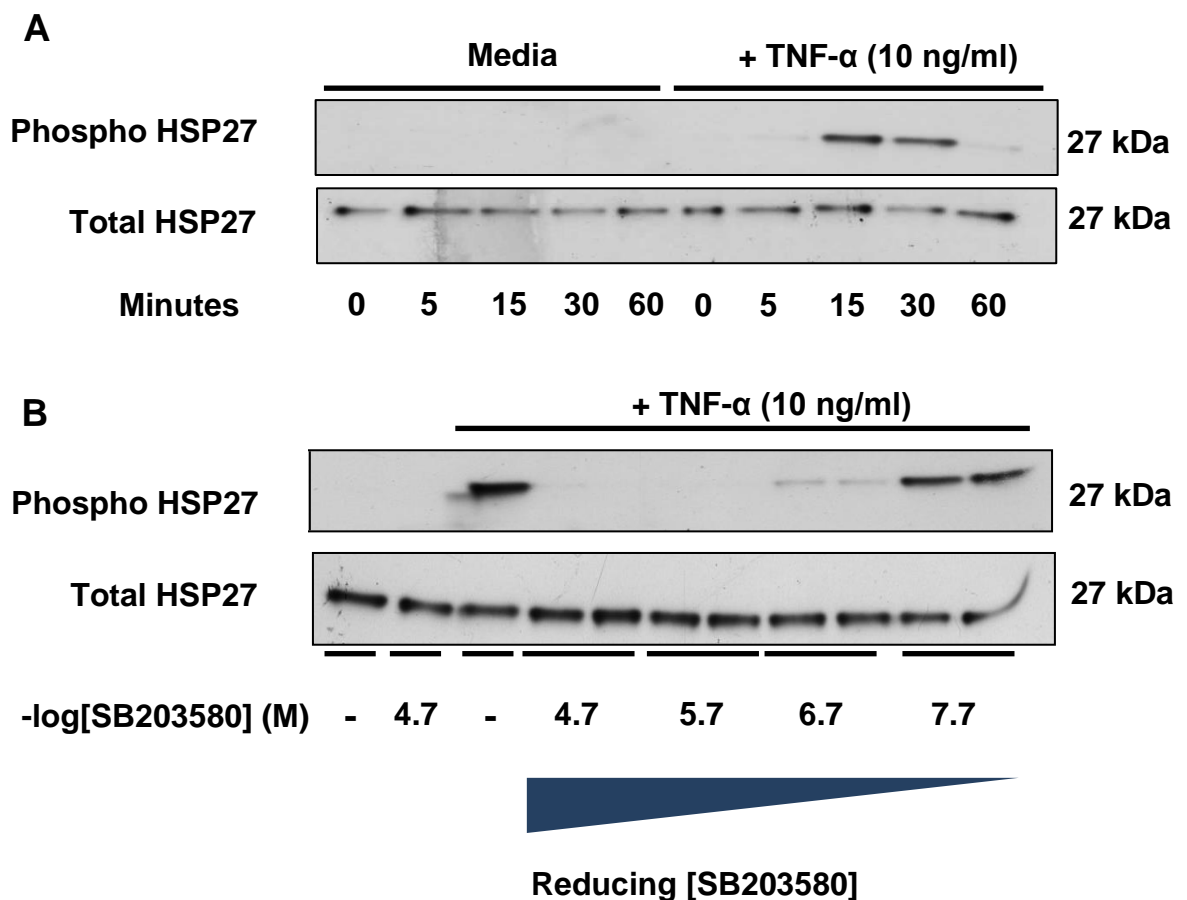


Figure 3.11.

TNF- α induces activation of p38 signalling (based on HSP27 phosphorylation) in a time-dependent manner and this effect is inhibited by SB203580 in pHLFs.

Panel (A) shows that TNF- α induces activation of the p38 signalling pathway in a time-dependent manner. pHLFs were exposed to control medium or TNF- α (10 ng/ml) for the indicated time periods. Activation of the p38 pathway was assessed by Western blotting examining phosphorylation of HSP27 (**upper panel, A**). Protein loading was confirmed by blotting with an anti-total HSP27 antibody (**lower panel, A**). **(B)** shows the effect of SB203580 on TNF- α -induced p38 signalling. Cells were pre-incubated with varying concentrations of SB203580 for 30 minutes prior to exposure to control medium or TNF- α (10 ng/ml) for 15 minutes. p38 signalling and protein loading was assessed as above. In this instance, the membrane was not stripped but a separate gel was run with identical protein concentrations (**lower panel, B**). Data are representative of three separate experiments performed.

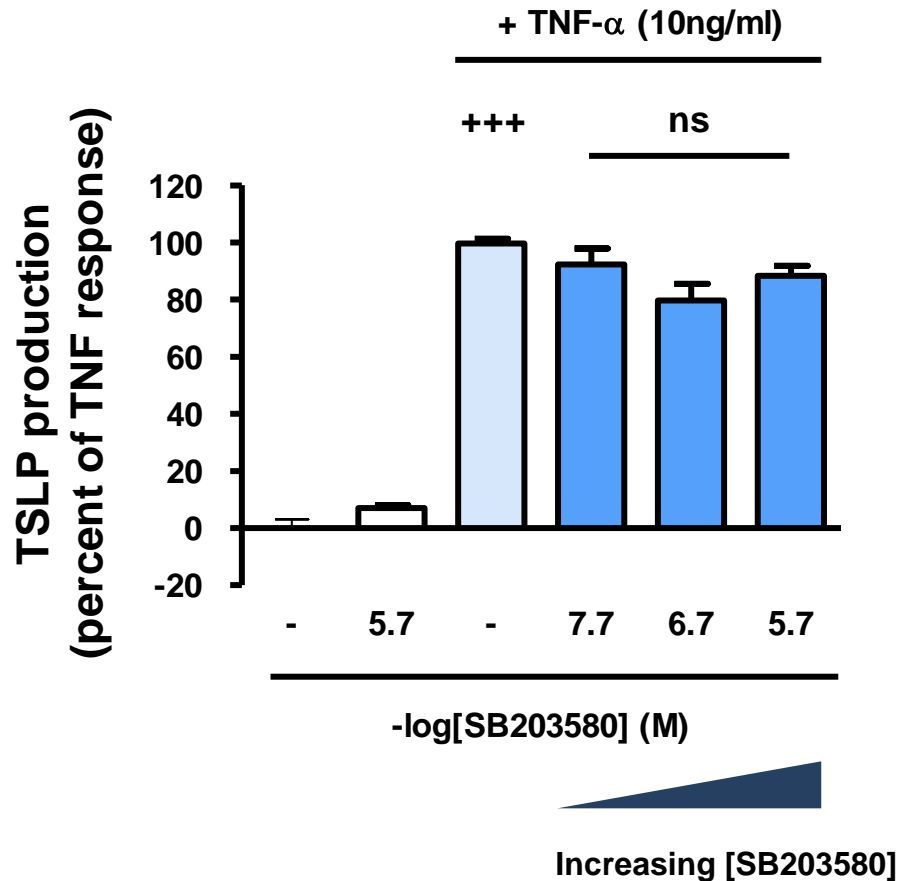


Figure 3.12.

TNF- α -induced TSLP protein release in pHLFs is not attenuated in a concentration-dependent manner by the p38 inhibitor, SB203580.

Figure shows the effect of the specific p38 inhibitor, SB203580, on TSLP protein release by pHLFs in response to TNF- α stimulation. Data are presented as a percentage of the maximal response obtained with TNF- α and drug vehicle alone (0.1% DMSO in DMEM). Cells were treated with increasing concentrations of SB203580 for 30 minutes prior to exposure to TNF- α (10 ng/ml) for 6 hours. Final concentrations of DMSO were kept constant for all experimental conditions. The first bar represents the TSLP response to control medium and drug vehicle alone. The second bar represents the highest concentration of SB203580 examined and shows that this compound has no effect on basal TSLP release. Negative log of the concentrations of SB203580 are presented. Data represent the mean \pm SEM, from triplicates, ns-non significant, comparison with TNF- α alone; +++ p <0.001, comparison with untreated cells; one-way ANOVA.

3.2.7.2. *The role of the ERK1/2 signalling pathway on TNF- α induced TSLP protein release in pHLFs*

I next examined the time-course of TNF- α -induced activation of the ERK1/2 signalling pathway. Cells were exposed to TNF- α (10 ng/ml) or control medium for varying durations (0 – 60 minutes) and p42/44 phosphorylation was examined by Western blotting. **Figure 3.13. (A)** shows that marked phosphorylation of p42/44 in pHLFs occurs following stimulation with TNF- α . An initial response was observed within 5 minutes followed by a return to levels similar to time-matched controls at 15 minutes. Thereafter, a further significant increase in p42/44 phosphorylation is observed which is maintained at 60 minutes following exposure to TNF- α .

The effect of U0126, a MEK1/2 inhibitor, on p42/44 phosphorylation was then examined. Cells, pre-incubated with increasing concentrations of U0126 (0 – 10 μ M) for 30 minutes, were exposed to TNF- α (10 ng/ml) for 30 minutes. **Figure 3.13. (B)** shows that U0126 inhibits TNF- α -induced phosphorylation of p42/44 in a concentration-dependent manner. Marked inhibition of p42/44 phosphorylation was observed for all concentrations of U0126 tested, though maximal inhibition, with almost complete loss of p42/44 phosphorylation was observed at concentrations of 3 μ M (-log 5.5) and above.

I next examined the effect of U0126 on TNF- α -induced TSLP protein release. pHLFs were pre-incubated with increasing concentrations of U0126 (0 – 10 μ M) for 30 minutes, prior to exposure to TNF- α (10 ng/ml) or control medium control medium for 6 hours. Conditioned media was then analysed for TSLP protein release by ELISA. **Figure 3.14.** shows that no attenuation in the TSLP signal was observed at U0126 concentrations of 3 μ M (-log 5.5 M) and below, although ~40% inhibition was observed at relatively high concentrations of 10 μ M (-log 5 M). Collectively, these data therefore suggest that the inhibition of the TSLP signal observed at these concentrations reflects off-target effects of U0126, and that ERK1/2 pathway does not play a role in mediating TNF- α -induced TSLP expression.

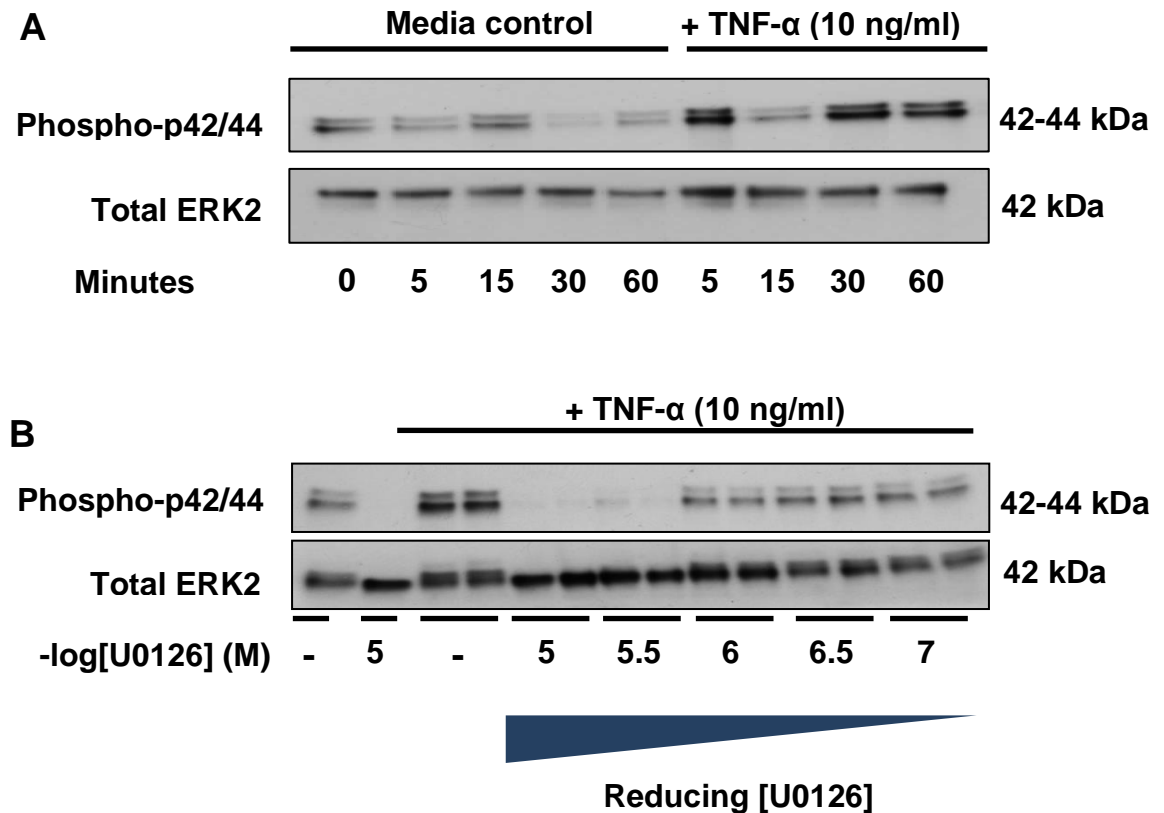


Figure 3.13.

TNF- α induces p42/44 phosphorylation in a time-dependent manner and this effect is inhibited by U0126 in pHLFs.

Panel (A) shows that TNF- α induces p42/44 phosphorylation in a time-dependent manner. pHLFs were exposed to control medium or TNF- α (10 ng/ml) for the indicated time periods. Phosphorylation of p42/44 was assessed by Western blotting using an anti-phospho-p42/44 antibody (**upper panel, A**). Protein loading was confirmed by blotting with an anti-total ERK2 antibody (**lower panel, A**). **Panel (B)** shows the effect of U0126, on TNF- α -induced p42/44 phosphorylation. Cells were pre-incubated with varying concentrations of U0126 for 30 minutes prior to exposure to control medium or TNF- α (10 ng/ml) for 30 minutes. p42/44 phosphorylation and protein loading was assessed as above. Data are representative of three separate experiments performed.

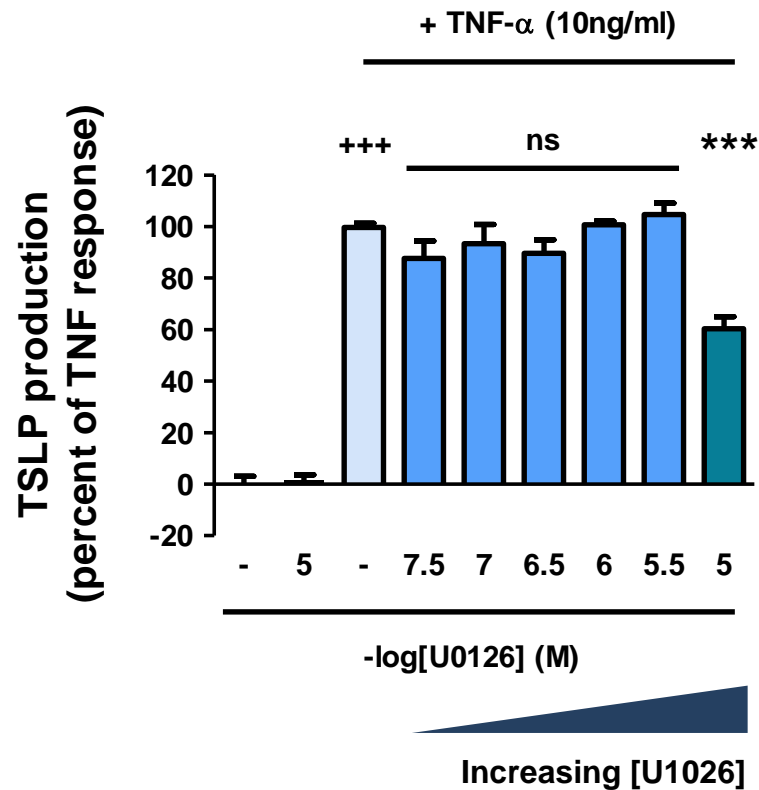


Figure 3.14.

TNF- α -induced TSLP protein release in pHLFs is not attenuated in a concentration-dependent manner by the MEK1/2 inhibitor U0126.

Figure shows the effects of the specific MEK 1/2 inhibitor, U0126, on TSLP protein release by pHLFs in response to TNF- α stimulation. Data are presented as a percentage of the maximal response obtained with TNF- α and drug vehicle alone (0.1% DMSO in DMEM). Cells were treated with increasing concentrations of U0126 for 30 minutes prior to exposure to TNF- α (10 ng/ml) for 6 hours. Final concentrations of DMSO were kept constant for all experimental conditions. The first bar represents the TSLP response to control medium and drug vehicle alone. The second bar represents the highest concentration of inhibitor examined and shows that this compound has no effect on basal TSLP release. Negative log of inhibitor concentrations are presented. Data represent the mean \pm SEM, from triplicates. ns-non-significant; *** $p < 0.001$, comparison with TNF- α alone; +++ $p < 0.001$, comparison with untreated cells; one-way ANOVA.

3.2.7.3. *The role of JNK in TNF- α induced TSLP expression*

The third major MAPK involved in activating AP-1 is JNK, and the following series of experiments were designed to examine the role of this kinase in mediating TNF- α -induced TSLP expression. For these studies, I used two structurally unrelated inhibitors of JNK activity, SP600125 and TI-JIP.

I first examined the time-course of TNF- α -induced phosphorylation of c-Jun in pHLFs. Cells were exposed to TNF- α (10 ng/ml) or control medium for varying durations (0 – 60 minutes) and c-Jun phosphorylation was assessed by Western blotting using an anti-phospho c-Jun antibody. **Figure 3.15.** shows that TNF- α induces marked phosphorylation of c-Jun from 15 minutes onwards and that this effect is maintained for at least 60 minutes.

I then assessed the effect of SP600125, an inhibitor of JNK activity, on TNF- α -induced c-Jun phosphorylation. Cells were pre-incubated with varying concentrations of SP600125 (0.3 – 10 μ M) before exposure to TNF- α (10 ng/ml) or control medium for 30 minutes. As shown in **Figure 3.16. (A-B)**, SP600125 significantly inhibits TNF- α -induced c-Jun phosphorylation in a concentration-dependent manner from 1 μ M (-log 6 M) (~25% inhibition) upwards.

Having demonstrated that SP600125 effectively inhibits TNF- α -induced c-Jun phosphorylation, I then examined the effect of this inhibitor on TNF- α -induced protein release. As previously, cells were exposed to varying concentrations of SP600125 (0 – 30 μ M) for 30 minutes prior to stimulation with TNF- α (10 ng/ml) for 6 hours and TSLP protein release into CM was measured by ELISA. **Figure 3.17.** shows that SP600125 significantly attenuated TNF- α -induced TSLP protein release in a concentration-dependent manner from 7 μ M (-log 7M) onwards upwards.

To strengthen the results obtained with SP600125, the effect of inhibiting JNK activity, using a structurally unrelated inhibitor, TI-JIP, on TNF- α -induced TSLP protein release was examined. As above, cells were pre-incubated with varying concentrations of TI-JIP for 30 minutes prior to exposure to TNF- α or control medium for 6 hours. **Figure 3.18.** shows that a significant inhibition of the TSLP signal was observed in a concentration-dependent manner from concentrations of TI-JIP of 0.1 μ M (-log 7 M) onwards.

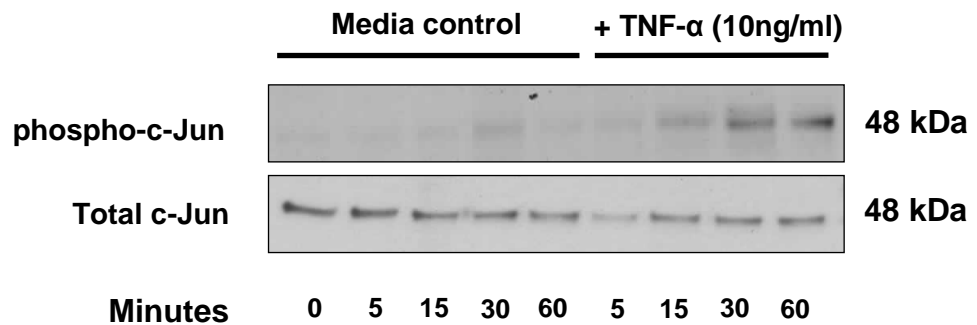


Figure 3.15.

Time course of TNF-α-induced phosphorylation of c-Jun in pHLFs.

Figure shows time-course data for the effect of TNF-α on pHLF c-Jun phosphorylation. pHLFs were exposed to control medium or TNF-α (10 ng/ml) for the indicated time periods (0-60 minutes). Phosphorylated c-Jun was assessed by Western blotting (**upper panel**). Protein loading was verified by blotting with an anti-total c-Jun antibody (**lower panel**). Data are representative of three independent experiments performed.

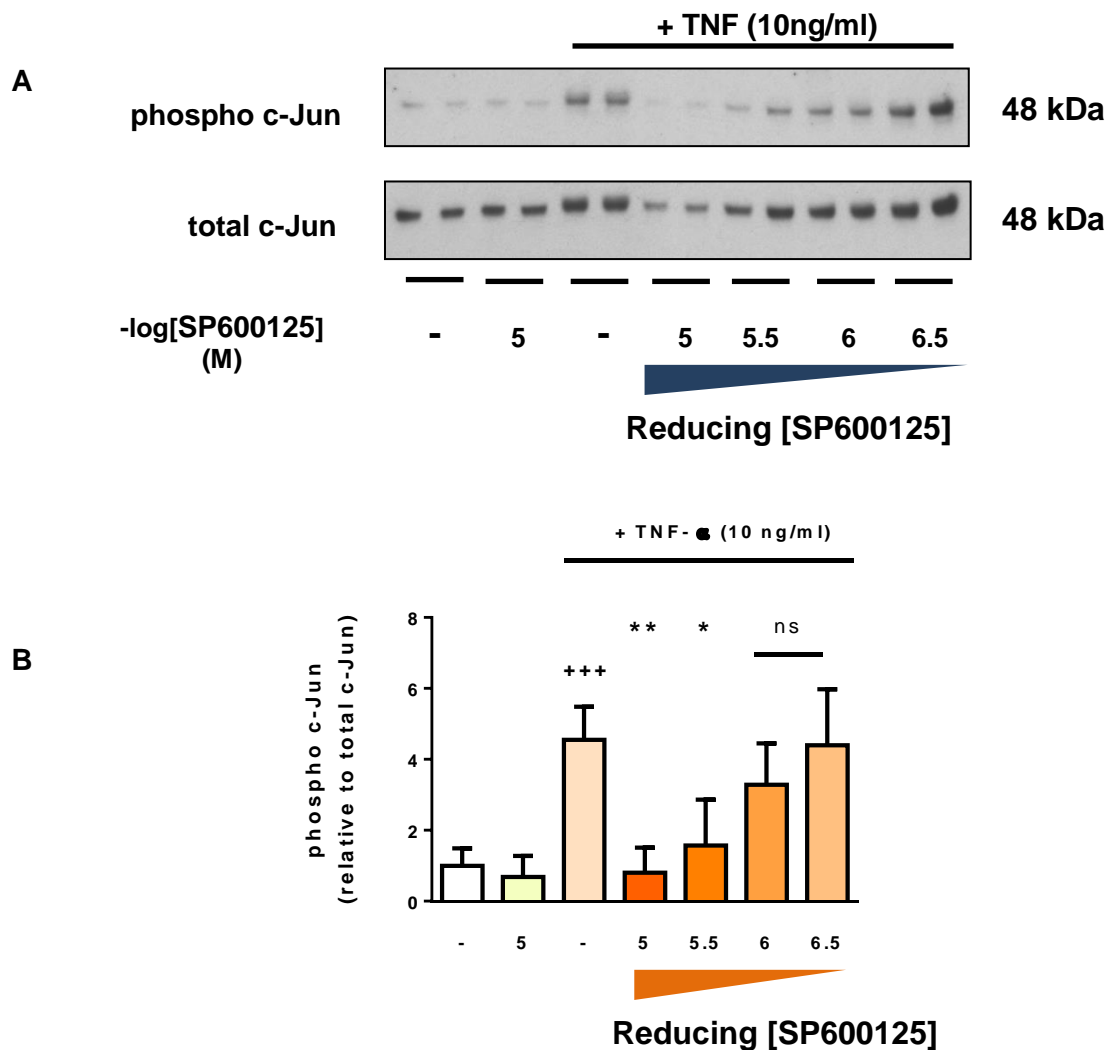


Figure 3.16.

TNF- α -induced c-Jun phosphorylation in pHLFs is attenuated in a concentration-dependent manner by the JNK inhibitor, SP600125.

Figure shows the effect of the specific JNK inhibitor, SP600125, on TNF- α -induced c-Jun phosphorylation. Cells were treated with control medium or with increasing concentrations of SP600125 for 30 minutes prior to exposure to TNF- α (10 ng/ml) for 30 minutes. Final concentrations of DMSO were kept constant for all experimental conditions (0.1% DMSO in DMEM). Phosphorylation of c-Jun (**upper panel, A**) and protein loading (**lower panel, A**) was assessed by Western blotting as previously described. The blots are representative of three independent experiments performed. The relative expression of phospho-c-Jun normalized to total c-Jun was determined by performing quantitative densitometry (**B**). Data represent the mean \pm SEM of duplicates from three independent experiments performed. +++ p <0.001, comparison with unstimulated cells; ns-non significant, * p <0.05 ** p <0.01, comparison to cells stimulated with TNF- α alone; one-way ANOVA.

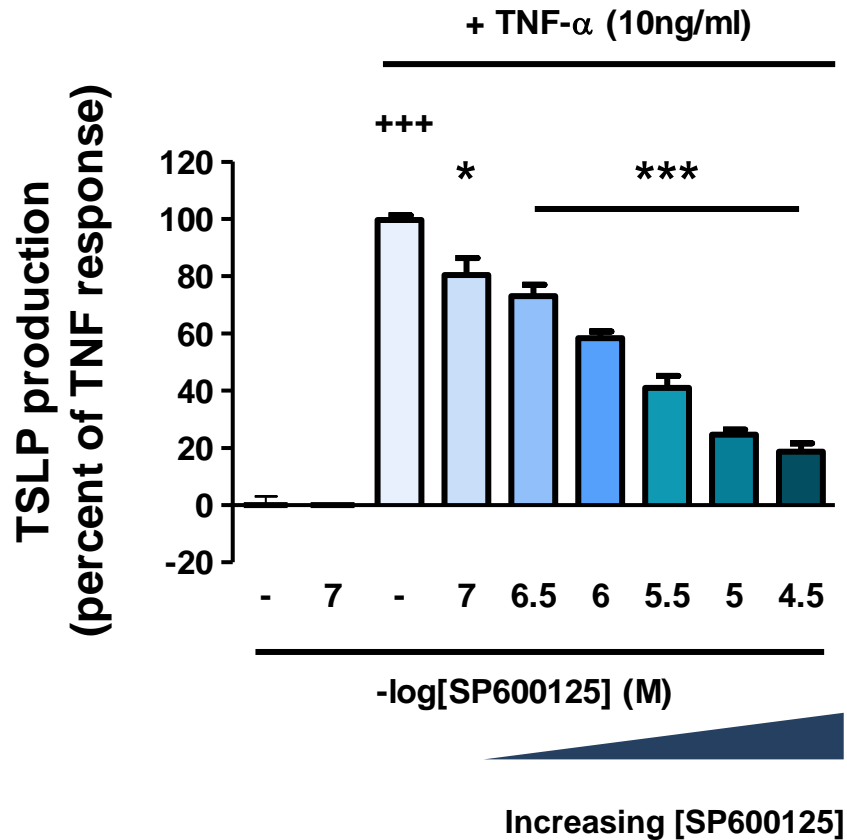


Figure 3.17.

TNF- α -induced TSLP protein release in pHLFs is attenuated in a concentration-dependent manner by the JNK inhibitor, SP600125.

Figure shows the effect of the specific JNK inhibitor, SP600125, on TSLP protein release by pHLFs in response to TNF- α stimulation. Data are presented as a percentage of the maximal response obtained with TNF- α and drug vehicle alone (0.1% DMSO in DMEM). Cells were treated with increasing concentrations of SP600125 for 30 minutes prior to exposure to TNF- α (10 ng/ml) for 6 hours. Final concentrations of DMSO were kept constant for all experimental conditions. The first bar represents the TSLP response to control medium and drug vehicle alone. The second bar represents the highest concentration of SP600125 examined and shows that this compound has no effect on basal TSLP release. Negative log of the concentrations of SP600125 are presented. Data represent the mean \pm SEM, from triplicates. * $p < 0.05$; *** $p < 0.001$, comparison with TNF- α alone; +++ $p < 0.001$, comparison with untreated cells; one-way ANOVA.

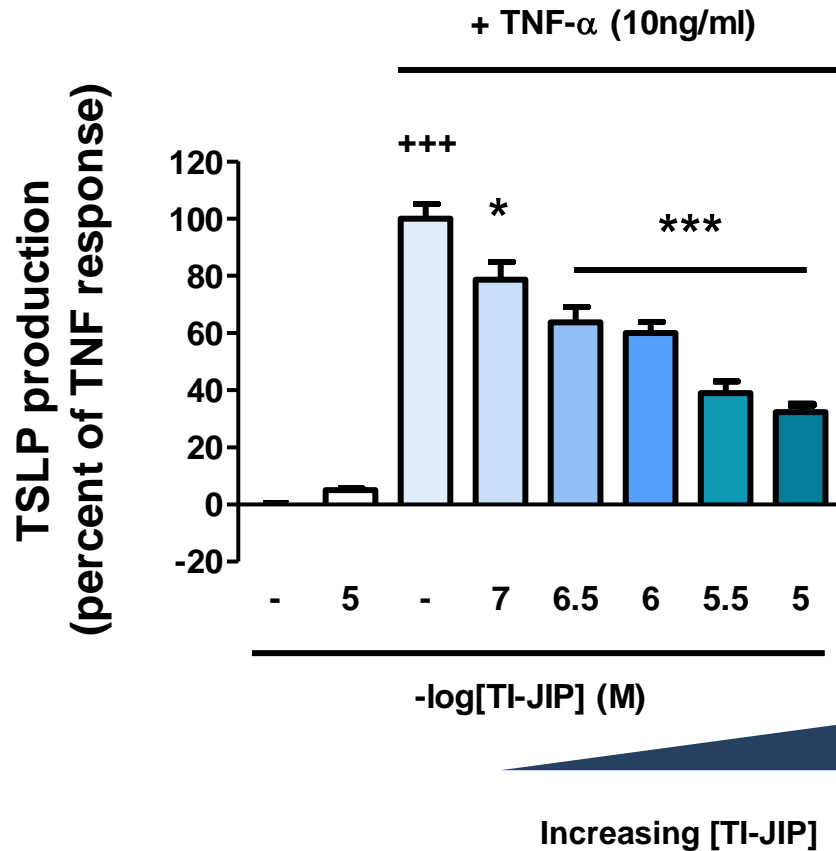


Figure 3.18.

TNF- α -induced TSLP protein release in pHLFs is attenuated in a concentration-dependent manner by the JNK inhibitor, TI-JIP.

Figure shows the effect of the specific JNK inhibitor, TI-JIP, on TSLP protein release by pHLFs in response to TNF- α stimulation. Data are presented as a percentage of the maximal response obtained with TNF- α and drug vehicle alone (0.1% DMSO in DMEM). Cells were treated with increasing concentrations of TI-JIP for 30 minutes prior to exposure to TNF- α (10 ng/ml) for 6 hours. Final concentrations of DMSO were kept constant for all experimental conditions. The first bar represents the TSLP response to control medium and drug vehicle alone. The second bar represents the highest concentration of TI-JIP examined and shows that this compound has no effect on basal TSLP release. Negative log of the concentrations of TI-JIP are presented. Data represent the mean \pm SEM, from triplicates. * $p < 0.05$; *** $p < 0.001$, comparison with TNF- α alone; +++ $p < 0.001$, comparison with untreated cells; one-way ANOVA.

The data presented above suggest that TNF- α induces TSLP protein release in a JNK / c-Jun dependent manner. To determine whether JNK mediates these effects by influencing TSLP mRNA levels, the effect of SP600125 on TNF- α -induced TSLP mRNA levels was examined. pHLFs were incubated with SP600125 (1 μ M) for 30 minutes prior to exposure to TNF- α (10 ng/ml) for 4 hours. TSLP mRNA levels were assessed as previously described in **Section 3.2.4.**

Figure 3.19. shows that at a concentration of 1 μ M, SP600125 significantly inhibited the TNF- α -induced increase in TSLP mRNA levels by ~ 70%. At this concentration, SP600125 inhibits TNF- α -induced TSLP protein release by ~40%. These data therefore suggest that TNF- α mediates its effects on TSLP protein and gene expression via the JNK/c-Jun signalling pathway.

To confirm the importance of the JNK / c-Jun signalling pathway in TNF- α -mediated TSLP production, a genetic approach was employed. pHLFs were transfected with siRNA targeted against c-Jun using a Gemini transfection reagent as described in **Materials and Methods**. Final concentrations of siRNA were 100 nM. Control cells were transfected with scrambled siRNA or mock-transfected for 24 hours. Following transfection, and a further 24 hours quiescence, cells were exposed to TNF- α (10 ng/ml) for 4 hours. Cell lysates were collected and analysed by Western blotting for c-Jun expression. Membranes were stripped and re-probed with anti-total ERK2 antibody to ensure equal protein loading. Conditioned media from the same wells were collected and analysed for TSLP protein release by ELISA. As shown in **Figure 3.20.**, transfection of pHLFs with c-Jun siRNA resulted in a significant knockdown of c-Jun expression at the protein level, compared to cells transfected with scrambled control siRNA, or cells which had been mock-transfected, at 4 hours following exposure to TNF- α . **Figure 3.21.** demonstrates this knockdown was associated with a significant (~50%) attenuation in TNF- α -induced TSLP protein release, confirming the importance of the JNK/c-Jun signalling pathway in mediating this response in pHLFs.

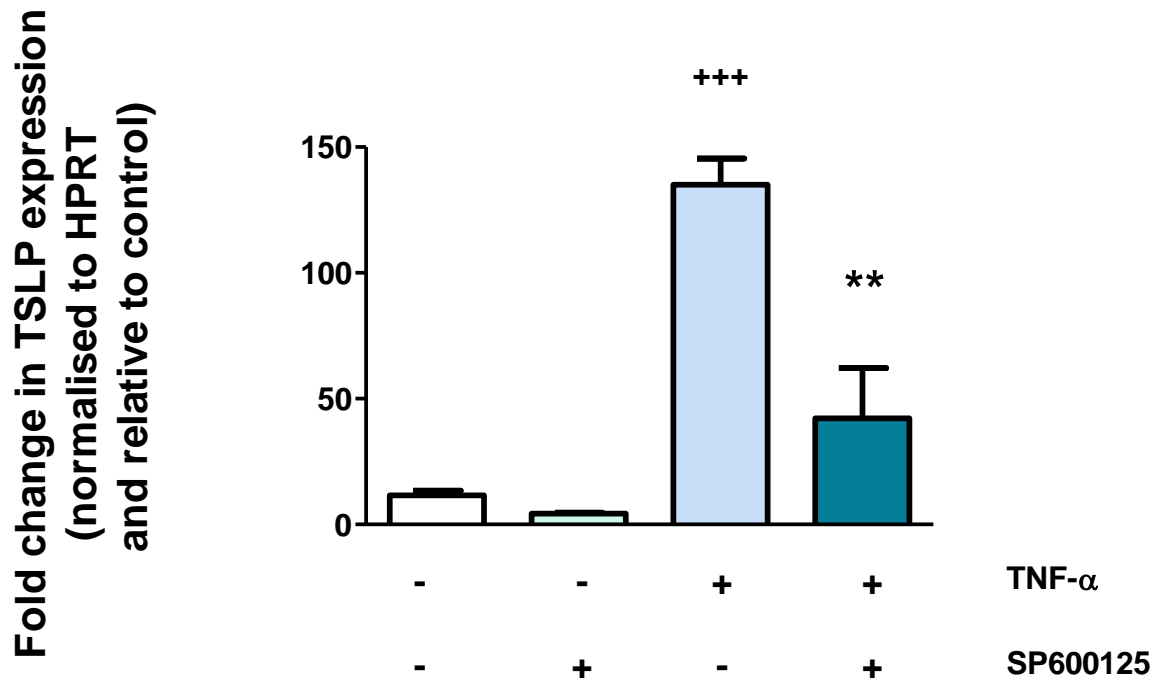
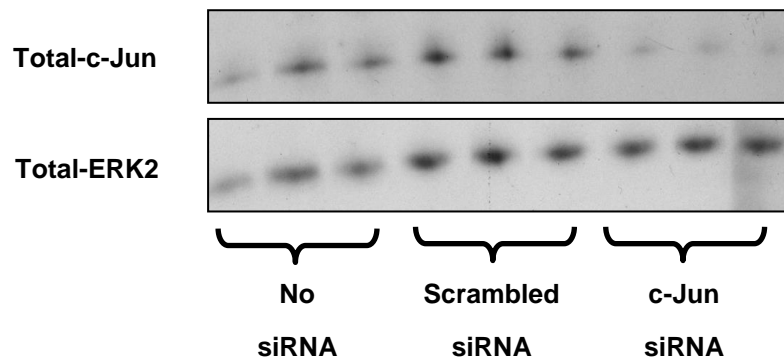


Figure 3.19.

Inhibition of TNF- α -induced upregulation of TSLP mRNA levels by the specific JNK inhibitor SP600125.

Figure shows the effect of the specific JNK inhibitor, SP600125, on TNF- α -induced TSLP mRNA levels. pHLFs were exposed to TNF- α (10 ng/ml) for 4 hours with or without pre-incubation with SP600125 (1 μ M) for 30 minutes. TSLP mRNA levels were determined by qRT-PCR. Data are presented as fold change relative to time zero following normalization to HPRT mRNA levels. Final concentrations of DMSO were kept constant for all experimental conditions (0.1% DMSO in DMEM). Data represent the mean \pm SEM, from triplicates. +++p<0.001, comparison with untreated cells; **p<0.01, comparison with TNF- α alone; one-way ANOVA.

A



B

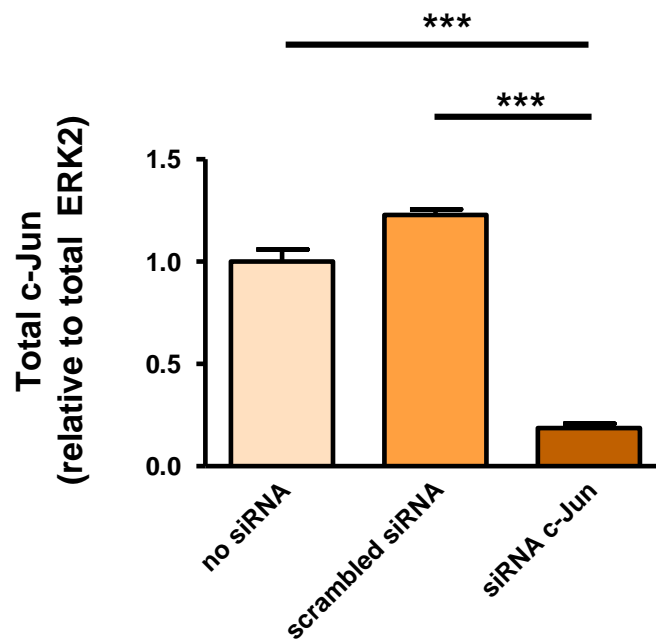


Figure 3.20.

Transfection of pHLFs with siRNA results in successful knock-down of c-Jun protein expression.

Figure shows that transfection of pHLFs with c-Jun specific siRNA results in knockdown of c-Jun expression at the protein level. 48 hours following mock-transfection, or transfection with scrambled or c-Jun specific siRNA (100nM), pHLFs were exposed to TNF- α (10 ng/ml) for 6 hours. Expression of c-Jun was assessed by Western blotting of cell lysates (**upper panel, A**); protein loading was confirmed by blotting with an anti-ERK2 antibody (**lower panel, A**). The relative expression of c-Jun normalized to total ERK2 was determined by performing quantitative densitometry (**panel B**). Data represent the mean \pm SEM from triplicates and are representative of three separate experiments performed. *** $p < 0.001$, comparison to mock-transfected cells; one-way ANOVA.

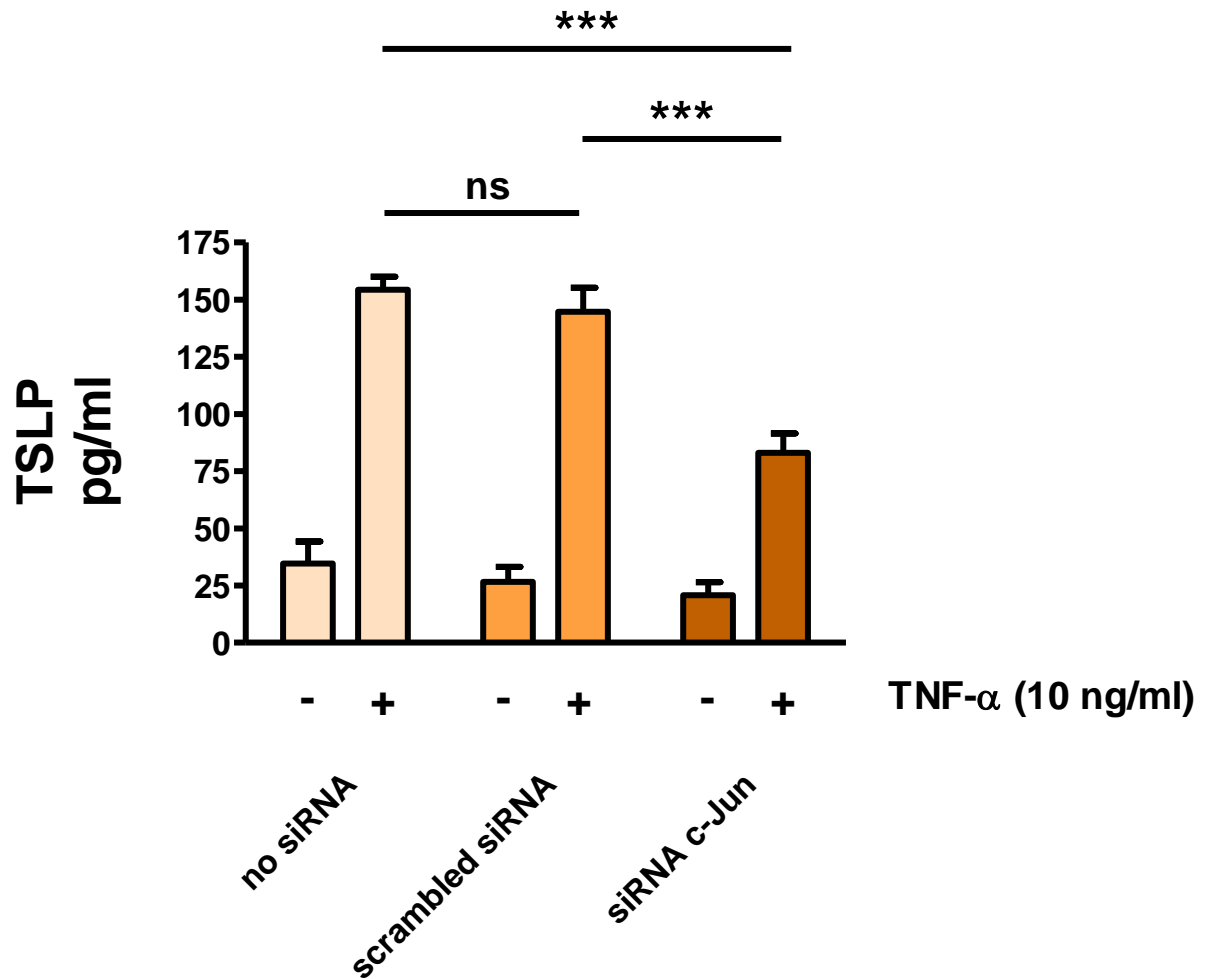


Figure 3.21.

Transfection of pHLFs with siRNA targeted against c-Jun results in an attenuation of TNF- α -induced TSLP protein release.

Figure shows the effect of transfection of pHLFs with c-Jun specific siRNA on TSLP-induced TSLP protein release. 48 hours after mock-transfection, or transfection with scrambled siRNA or c-Jun-specific siRNA (100 nM), pHLFs were exposed to TNF- α (10 ng/ml) or control medium for 6 hours. Expression of c-Jun was assessed by Western blotting as shown in **Figure 3.24**. Time-matched supernatants were collected and analysed for TSLP protein release by ELISA. The amount of secreted TSLP is expressed as pg/ml, and each value represents the mean \pm SEM, from triplicates. ns-non significant; *** $p < 0.001$ comparison with mock-transfected cells; two-way ANOVA.

3.2.8. Summary

The results described in this section, examining the expression of TSLP by human lung epithelial cells and primary adult human lung fibroblasts (pHLFs), showed that:

- TNF- α does not induce TSLP production in primary type II alveolar epithelial cells derived from normal human lung, nor in the A549 epithelial cell line;
- TNF- α induces TSLP protein production and release in a concentration and time-dependent manner in pHLFs;
- TNF- α upregulates TSLP mRNA levels in pHLFs in a time-dependent manner;
- TNF- α increases the long-form splice variant TSLP mRNA levels in pHLFs; no effect on short-form splice variant TSLP mRNA levels is observed;
- The NF κ B, p38 and ERK1/2 pathways do not seem to play a role in mediating TNF- α induced TSLP protein release by pHLFs;
- The JNK / c-Jun pathway mediates TNF- α induced TSLP expression by pHLFs.

In conclusion, the data presented indicate that TNF- α induces TSLP expression via the JNK / c-Jun pathway in pHLFs. Moreover, the data suggest that this occurs via transcription of the long- splice variant of TSLP.

3.3. The effect of TSLP stimulation of primary human lung fibroblasts (pHLFs)

3.3.1. Introduction

The data presented in **Section 3.1.** demonstrates strong immunoreactivity for TSLPR associated with lung fibroblasts in IPF, findings which have not previously been reported. However, it is increasingly recognised that fibroblasts may serve as key immunoregulatory cells following tissue injury, both via the generation of chemokines / cytokines (Smith *et al.*, 1997) and through their direct cell – cell interactions with immune cells (Yamamura *et al.*, 2001). These observations led to the hypothesis that lung fibroblasts may represent a potential cellular target for TSLP and that the response of these cells to stimulation with TSLP would reflect their immunoregulatory capacity. The following section addresses this hypothesis by assessing expression of a TSLP/TSLPR signalling axis by pHLFs and examining the effect of TSLP on pHLFs *in vitro*.

3.3.2. Expression of the TSLP receptor by lung fibroblasts *in vitro*.

Expression of both components of the TSLP receptor complex was initially assessed by RT-PCR using RNA extracted from pHLFs and primary murine lung fibroblasts (pMLFs); primers were custom-designed as described in **Materials and Methods**. As can be seen in **Figure 3.22.**, prominent PCR products were obtained for TSLPR α and IL-7R α in both pHLFs and pMLFs. No-cDNA template controls served as negative controls in each adjacent lane, confirming that the PCR products were not the result of primer dimerization. These data demonstrate that lung fibroblasts have the potential to express the TSLP receptor complex.

I next examined protein expression of the relevant receptor components by pHLFs *in vitro* by fluorescent immunocytochemistry. **Figure 3.23.** shows representative images of pHLFs incubated with anti-TSLPR and anti-IL-7R α antibodies and counterstained with DAPI to enable visualisation of the nucleus, as described in **Materials and Methods**. Positive staining for both TSLPR α and IL-R α chains was observed for pHLFs (**panels A-B**). Overlay of images demonstrated a strong degree of overlap, suggestive of co-localisation of these 2 receptor chains (**panel C**). Signal specificity was demonstrated by loss of fluorescent signal upon substitution of anti-TSLPR and anti-IL-7R α antibodies with isotype control antibodies (**panels D-E**). Separate chamber slides were incubated with either anti-TSLPR α or anti-IL7-R α antibodies alone. Images were

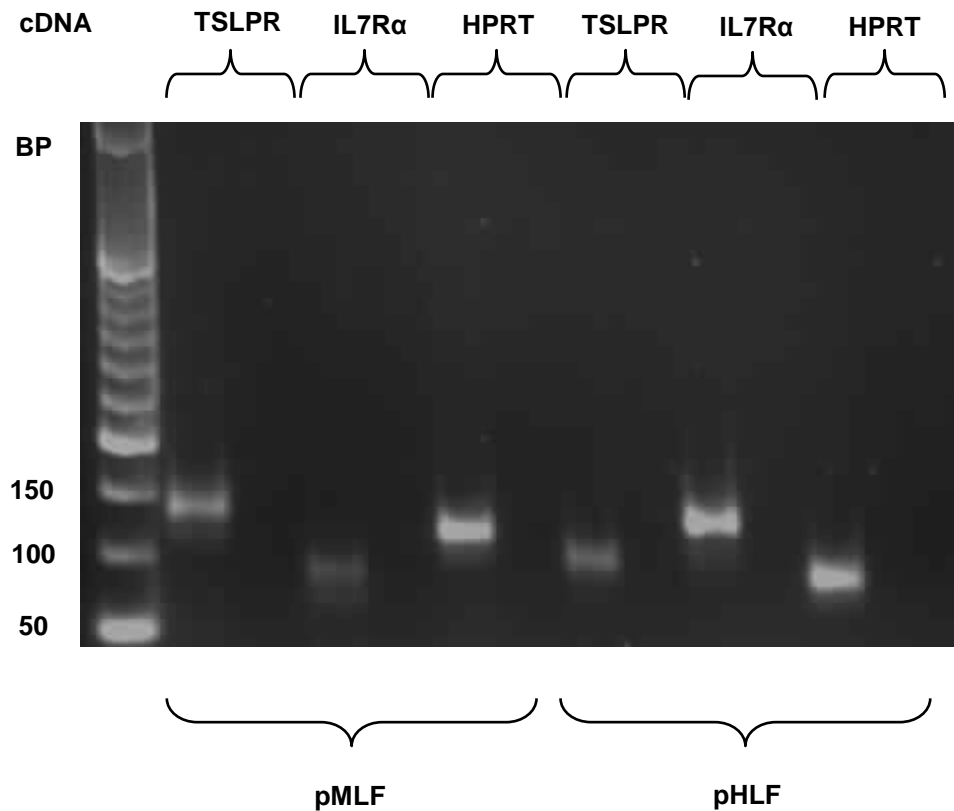


Figure 3.22.

Expression of TSLPR and IL-7R α in primary murine and human lung fibroblasts.

Figure shows the expression of TSLPR and IL-7R α , as analysed by RT-PCR in whole cell lysates of primary murine lung fibroblasts (pMLFs) and primary human lung fibroblasts (pHLFs). 1 μ g of cDNA was used for each set of primers and the same volume of final PCR products was run in a 1% agarose gel containing the nucleic acid gel stain, GelRed (1:10,000). No cDNA templates were run in each adjacent lane as negative controls, and expression of the housekeeping gene HPRT, specific for each species, was analysed as a positive control. (BP – base pairs)

captured for each such incubation in both fluorescent channels, and overlaid to ensure that any positive signal observed was not an artefact due to cross-over from the alternative channel. As can be seen in **panels F-G**, no such overlap was observed, further confirming the specificity of the signals obtained for each antibody targeted against its respective receptor chain. Collectively, these data allowed me to conclude that pHLFs express both components of the TSLP receptor complex.

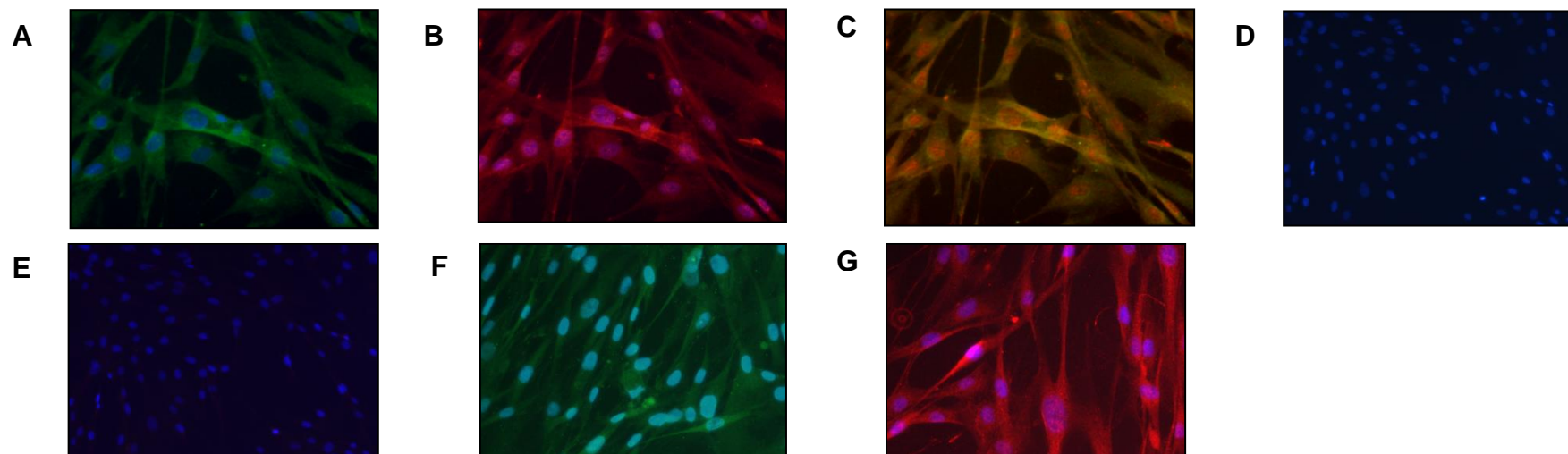


Figure 3.23.

Primary human lung fibroblasts express both components of the TSLP receptor complex.

pHLFs were grown to 80% confluence and TSLPR and IL7R α expression was analysed by means of dual immunofluorescence as described in *Materials and Methods*. pHLFs were stained with anti-TSLPR-AF488 (**A**), anti-IL7R α -AF555 (**B**), and counterstained with DAPI to enable nuclear localisation. Staining overlap is demonstrated in panel (**C**). Substitution of anti-TSLPR or anti-IL7R α antibodies with appropriately matched isotype control antibodies (**D** and **E** respectively) eliminated the fluorescent staining. (**F**) represents an overlay of images obtained in both AF488 and AF555 channels following incubation with anti-TSLPR-AF488 only. (**G**) represents an overlay of images obtained in the same channels following incubation with anti-IL7R α -AF555 only. Original magnifications x20.

3.3.3. Effect of TSLP on pHLF chemokine production

Having demonstrated that pHLFs constitutively express both components of the TSLP receptor, I next examined whether the observed co-localisation of these chains reflected functionality of this receptor. The immunoregulatory role of fibroblasts may be ascribed to the elaboration of soluble factors, in particular chemokines (Buckley *et al.*, 2001). Therefore, to evaluate this potential role, the effect of TSLP on pHLF chemokine expression was examined. Cells were exposed to TSLP (1 ng/ml) or control medium for varying durations (1, 2, 4, 8 and 24 hours). Conditioned media (CM) was collected and analysed for chemokine release using the MSD® electroluminescence detection platform. This platform is a multiplex immunoassay system permitting analysis of multiple chemokines simultaneously. The choice of chemokines assayed reflected those which have previously been implicated in the pathogenesis of pulmonary fibrosis, including CCL2, CCL7 and CCL22, as discussed in **Section 1.7.4.**, but also included eotaxin and IP-10, chemokines elaborated by fibroblasts which are chemotactic for immune cells, including eosinophils (Wenzel *et al.*, 2002), neutrophils (Hammond *et al.*, 1995) and T-cells respectively (Dufour *et al.*, 2002). As can be seen from **Figure 3.24. (Panel A)**, TSLP promoted significant CCL2 protein release compared to control (1.5-fold increase at 24 hours). CCL2 protein levels were also observed to accumulate in CM of untreated control pHLFs, confirming previous reports of basal production of this chemokine (Deng *et al.*, 2008), though no further increase was observed in these studies after 8 hours. Although pHLFs were found to produce a number of other mediators, no other chemokine release was demonstrated over this time course following exposure to TSLP (**Figure 3.24. Panels B-E**). Nonetheless, these data strongly suggest that pHLFs possess a functional TSLP receptor, and that they respond to TSLP by upregulating expression of CCL2.

3.3.4. Effect of TSLP on pHLF CCL2 expression

The previous findings support the notion that lung fibroblasts may represent an important immunomodulatory cell by releasing CCL2 in response to TSLP. To determine if TSLP exerts these effects by influencing *Ccl2* gene expression, the effect of TSLP on CCL2 mRNA levels was assessed by qRT-PCR. Cells were exposed to TSLP (1 ng/ml) or control medium for varying durations (0 – 24 hours) and cell lysates collected for measurement of mRNA levels. **Figure 3.25.** demonstrates that exposure of pHLFs to TSLP resulted in a significant increase

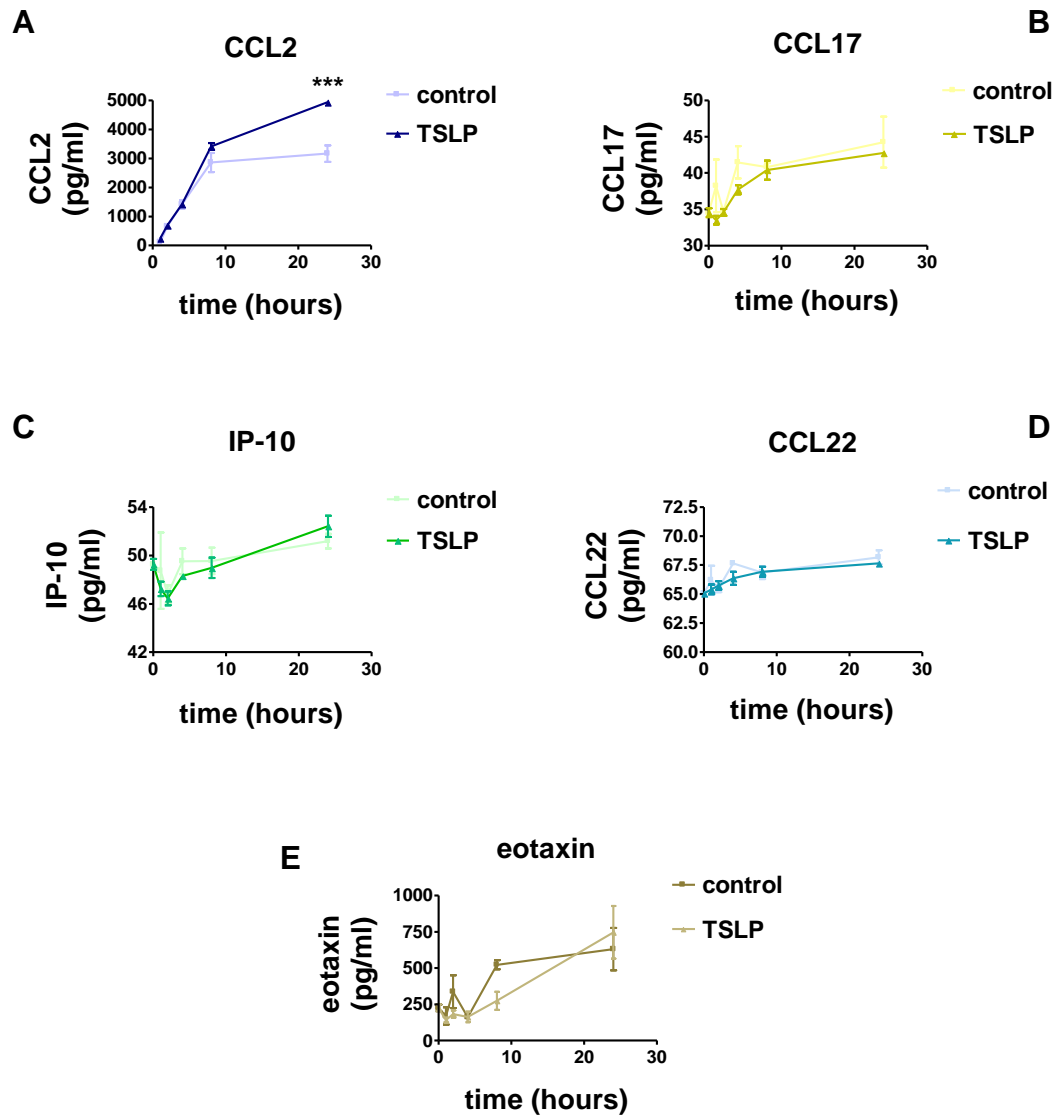


Figure 3.24.

Effect of TSLP on chemokine expression by pHLFS.

Figure shows the time-course data for the effect of TSLP on pHLF chemokine protein release into conditioned media. pHLFs were exposed to TSLP (1 ng/ml) or control medium for varying durations (0-24 hours). Conditioned media was analysed for CCL2 **(A)**; CCL17 **(B)**; IP-10 **(C)**; CCL22 **(D)** and eotaxin **(E)** protein release using the MSD ® electrochemoluminescence detection platform. The amount of secreted chemokine is expressed as pg/ml and each value represents the mean \pm SEM, from triplicates. *** $p < 0.001$, comparison with time point-matched media control; two-way ANOVA.

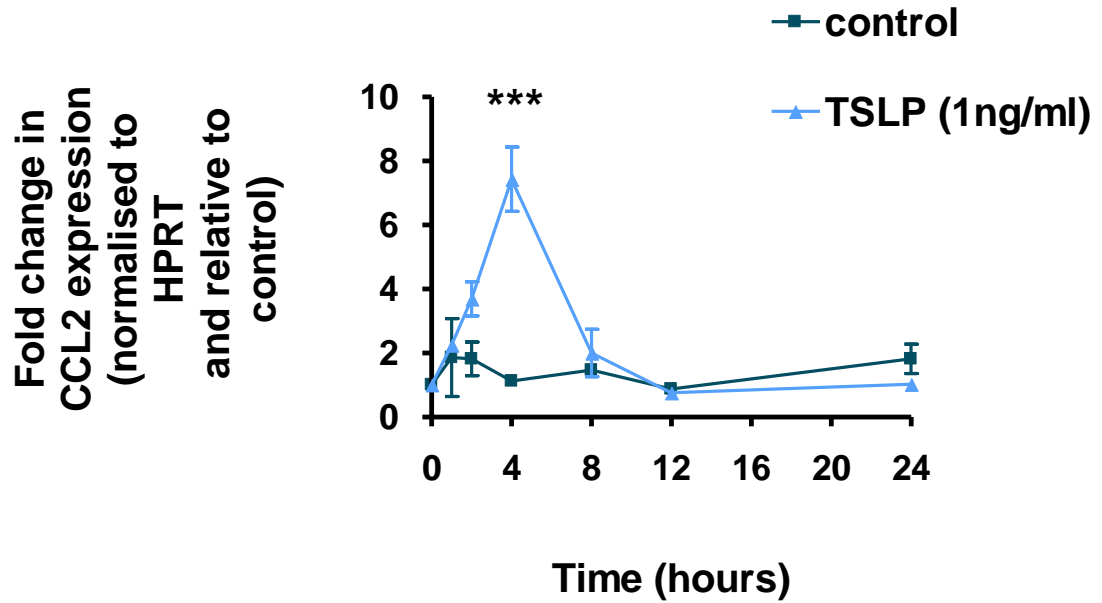


Figure 3.25.

TSLP increases CCL2 mRNA levels in pHLFs.

Figure shows time-course data for the effect of TSLP on pHLF CCL2 mRNA levels. pHLFs were exposed to serum-free control medium or TSLP (1 ng/ml) for varying durations from 0-24 hours. TSLP mRNA levels at each time point were assessed by qRT-PCR. Data are expressed as fold-change relative to time zero following normalization to HPRT mRNA levels (mean \pm SEM, from triplicates). *** $p < 0.001$, comparison with time-matched media controls; two-way ANOVA.

in CCL2 mRNA levels at 4 hours (~7-fold relative to control). CCL2 mRNA levels declined after this time-point and returned to baseline by 24 hours.

TSLP-induced CCL2 expression at the protein level was further confirmed in an independent series of time-course and concentration-response experiments. Cells were exposed to varying concentrations of TSLP and CCL2 protein release into CM was analysed by ELISA at 0, 1, 2, 4, 8, 12 and 24 hours. **Figure 3.26.** shows TSLP (1 ng/ml) promotes CCL2 protein release in a time-dependent manner. Peak CCL2 protein levels were reached 12 hours after exposure (1674 pg/ml \pm 31 vs 890 pg/ml \pm 13) with no further increase observed thereafter. Since the time-course studies had demonstrated a significant increase in TSLP-induced CCL2 protein release at 6 hours, this time point was chosen for future concentration-response studies. As can be seen from **Figure 3.27.**, TSLP promotes CCL2 protein release in a concentration-dependent manner from 0.03 ng/ml (-log 10.5 g/l) onwards. A 5-fold increase in CCL2 protein levels, relative to control, was observed with TSLP at a concentration of 1 ng/ml (-log -9 g/l) with no further increment in CCL2 protein release at higher concentrations (data not shown).

CCL2 expression is exquisitely sensitive to endotoxin contamination. To confirm that the observed upregulation of CCL2 protein release in pHLFs exposed to TSLP was not the result of such contamination, the effect of neutralising TSLP, using a commercially available polyclonal sheep IgG anti-TSLP antibody, on this response was examined. Cells were exposed to TSLP (1 ng/ml) which had been pre-incubated with varying concentrations of anti-TSLP antibody (0 – 1 μ g/ml), isotype control antibody (1 μ g/ml), control medium alone or respective antibodies alone (1 μ g/ml). CM was analysed for CCL2 protein release at 6 hours by ELISA. As can be seen from **Figure 3.28.**, CCL2 production was attenuated in cells exposed to TSLP which had been pre-incubated with anti-TSLP antibody, in a concentration-dependent manner from 0.1 μ g/ml (-log 7 g/l) onwards. The TSLP-induced CCL2 response was almost completely blocked at an anti-TSLP antibody concentration of 1 μ g/ml (-log 6 g/l). The ND₅₀ of this antibody for this response was determined to be 0.25 μ g/ml. Pre-incubation of TSLP with a maximal concentration of isotype control antibody did not result in any inhibition of the CCL2 response, and exposure to antibodies alone did not result in any significant CCL2 protein release. These data lend strong support to the conclusion that the effect of TSLP on CCL2 protein release is not mediated via a contaminant in the TSLP preparation.

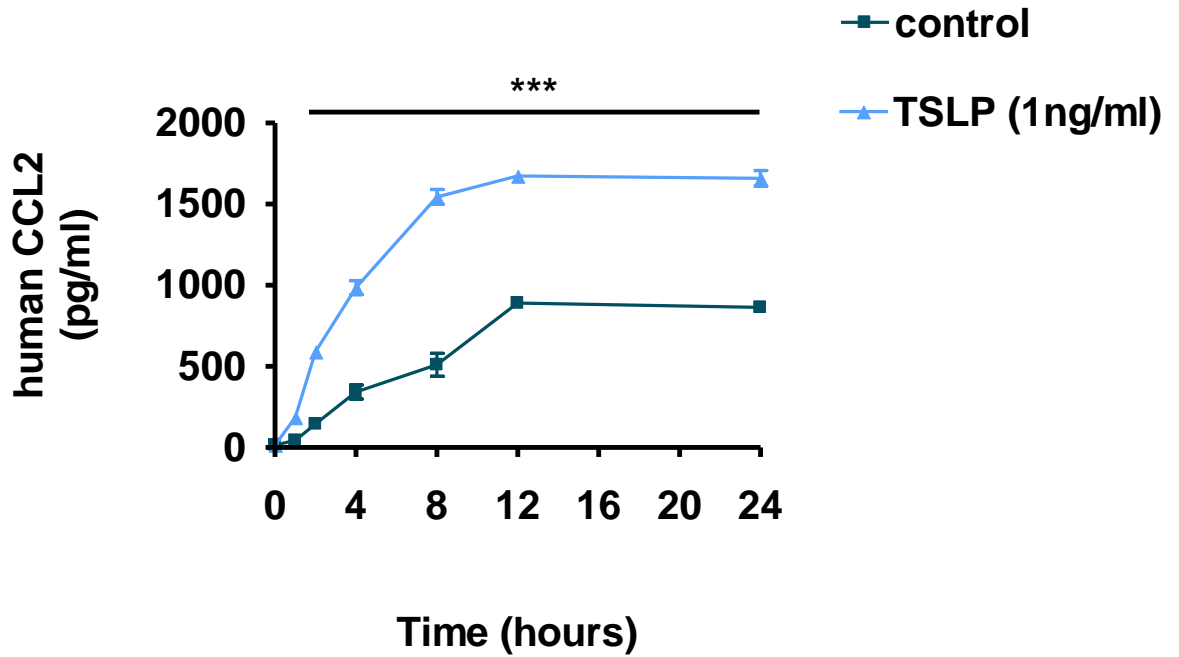


Figure 3.26.

TSLP stimulates pHLF CCL2 protein release in a time-dependent manner.

Figure shows time course data for the effect of TSLP on pHLF CCL2 protein release into conditioned media. pHLFs were exposed to TSLP (1 ng/ml) for varying durations (0-24 hours). Cell cultures supernatants were analysed for CCL2 protein release by ELISA. The amount of secreted CCL2 is expressed as pg/ml, and each value represents the mean \pm SEM, from triplicates. *** $p < 0.001$, comparison with time-point matched media control; two-way ANOVA.

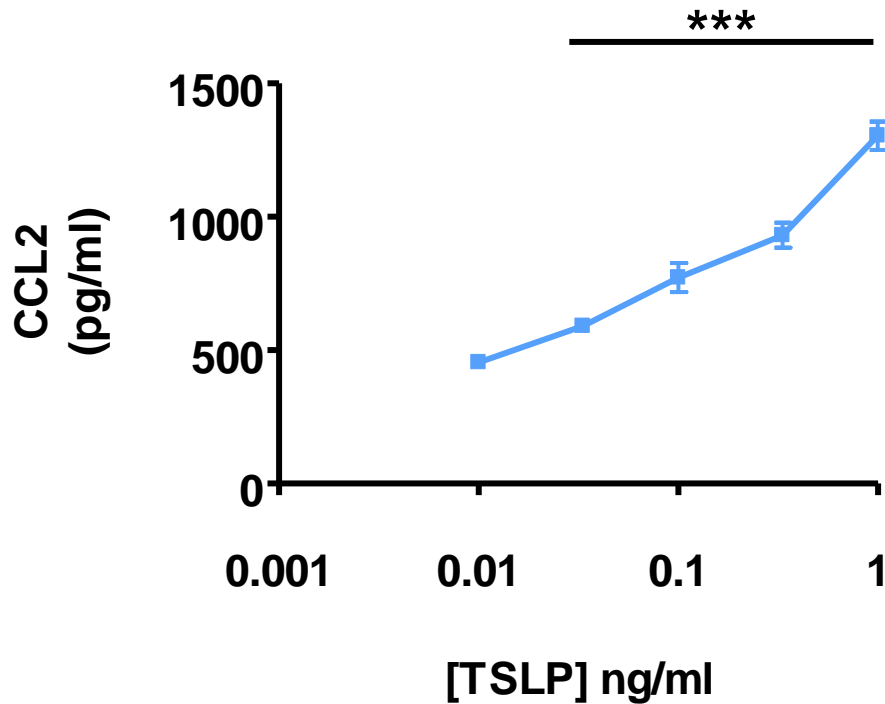


Figure 3.27.

TSLP stimulates pHLF CCL2 protein release in a concentration-dependent manner.

Figure shows concentration-response data for the effect of TSLP on pHLF CCL2 protein release into conditioned media. pHLFs were exposed to serum-free control medium or TSLP (0.01 – 1 ng/ml) for 6 hours. Cell culture supernatants were analysed for CCL2 protein release by ELISA. The amount of secreted CCL2 is expressed as pg/ml, and each value represents the mean \pm SEM, from triplicates. *** $p < 0.001$, comparison with unstimulated cells; one-way ANOVA.

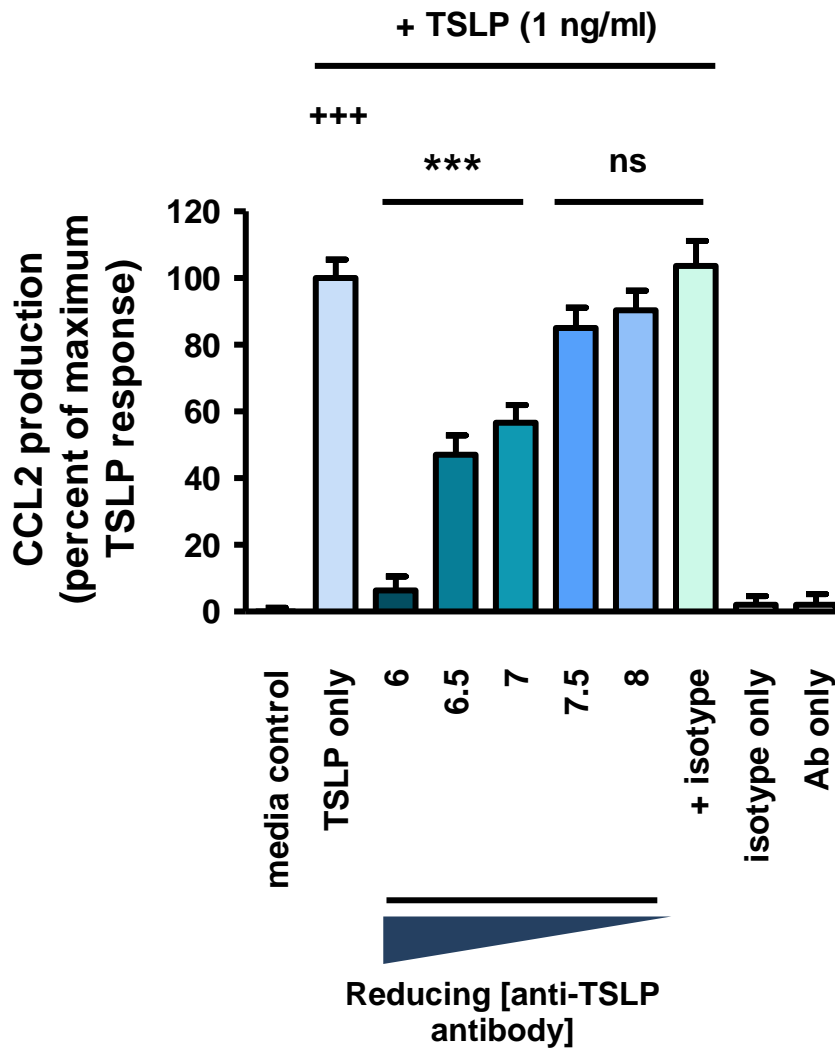


Figure 3.28.

TSLP-induced CCL2 protein release by pHLFs is inhibited by an anti-TSLP antibody.

Figure shows the effect of an anti-TSLP antibody on TSLP-induced CCL2 protein release. Data are presented as a percentage of the maximal response obtained with TSLP only. pHLFs were exposed for 6 hours to control medium; isotype control antibody only (1 µg/ml); anti-TSLP antibody only (1 µg/ml); TSLP (1 ng/ml) pre-incubated with isotype control antibody (1 µg/ml) or varying concentrations of anti-TSLP antibody (for 2 hours). Conditioned media were analysed for CCL2 protein release by ELISA. The first bar represents the response to control medium alone. The second bar represents the response to TSLP only. The final bars represent the response to isotype control and anti-TSLP antibodies respectively (1 µg/ml), and show that these antibodies had no effect on basal CCL2 release. Negative log of the concentrations of anti-TSLP antibody (g/l) are presented. Data represent the mean ± SEM, from triplicates. +++p<0.001, comparison with control medium; ns-non significant, ***p<0.001, comparison with TSLP alone.

3.3.5. *Effect of TSLP on signal transducer and activator of transcription (STAT) phosphorylation in pHLFs*

Having demonstrated that TSLP upregulates expression of CCL2 in pHLFs, the next series of experiments was designed to investigate further the signalling mechanisms downstream of TSLP receptor ligation. Ligand-induced cross-linking of TSLPR α and IL-7R α has previously been demonstrated to result in functional activation of both STAT5 and STAT3 in a number of different cell types (Isaksen *et al.*, 1999; Wohlmann *et al.*, 2010). Moreover, STAT3 has recently been reported to mediate transcription of CCL2 (Dauer *et al.*, 2005). In light of these data, the following series of experiments examined the relative roles of STAT5 and STAT3 in mediating TSLP-induced CCL2 expression.

I first examined the effect of TSLP on STAT5 and STAT3 phosphorylation in pHLFs. Cells were exposed to TSLP (1 ng/ml) or control medium for varying durations (0 – 60 minutes) and cell lysates were assessed by Western blotting using anti-phospho STAT5 or anti-phospho STAT3 antibodies. As can be seen in **Figure 3.29. (A)**, no induction of STAT5 phosphorylation was observed in either control cells or pHLFs exposed to TSLP over a period of 1 hour. In contrast, TSLP induced the phosphorylation of STAT3 in a time-dependent manner (**Figure 3.29. (B)**) from 15 minutes onwards which was maintained for at least 60 minutes. No phosphorylation of STAT3 was observed in control cells exposed to media alone.

To confirm the specificity of this response for TSLP, I examined the effect of neutralising TSLP on the STAT3 phosphorylation, using the same anti-TSLP antibody as described in **Section 3.3.4**. Cells were exposed to the same experimental conditions and STAT3 phosphorylation was assessed by Western blotting. As seen in **Figure 3.30.**, pre-incubation of TSLP with anti-TSLP antibody resulted in a concentration-dependent inhibition of TSLP-induced STAT3 phosphorylation assessed at 30 minutes at antibody concentrations of 0.03 μ g/ml onwards. Abrogation of TSLP-induced STAT3 phosphorylation was observed in the presence of anti-TSLP antibody concentrations of 1 μ g/ml. These data confirm that the observed phosphorylation of STAT3 in pHLFs exposed to TSLP is mediated by TSLP rather than a contaminant. Collectively, these findings demonstrate that TSLP induces STAT3 but not STAT5 phosphorylation in pHLFs.

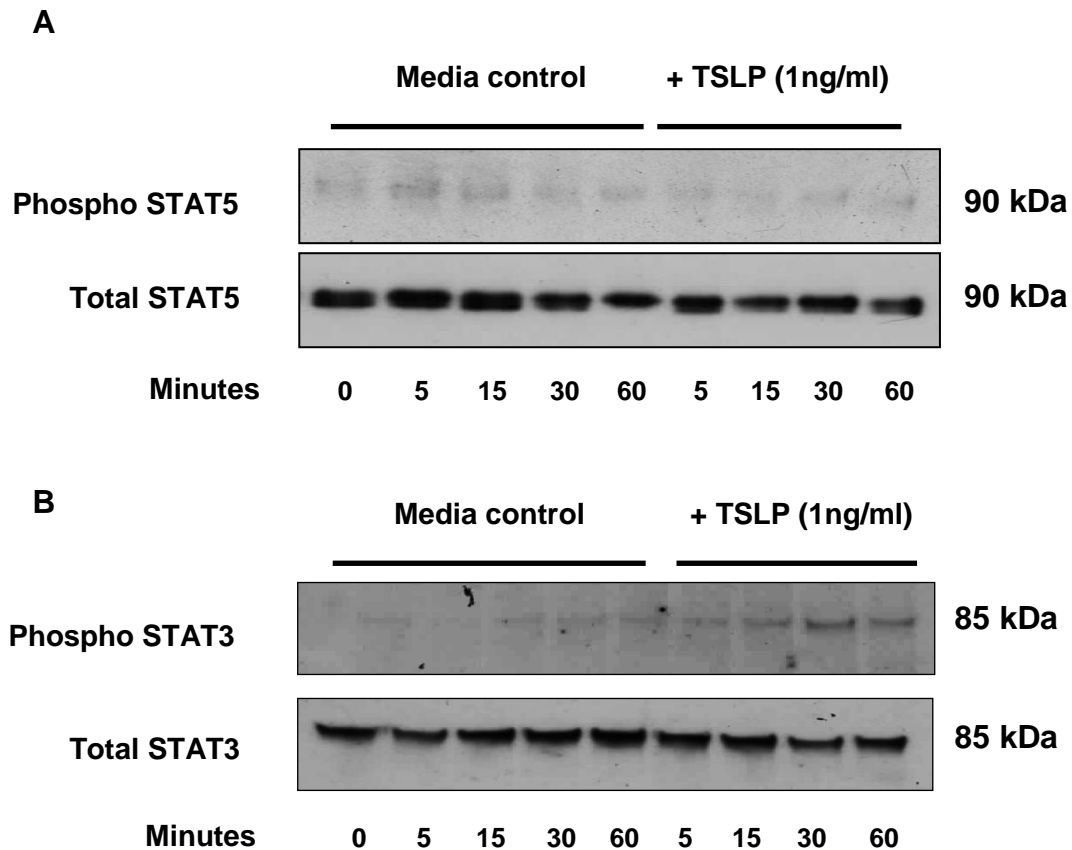


Figure 3.29.

TSLP induces STAT3, but not STAT5 phosphorylation in pHLFs.

Panel (A) shows time-course data for the effect of TSLP on pHLF STAT5 phosphorylation. pHLFs were exposed to control medium or TSLP (1 ng/ml) for the indicated time periods (0-60 minutes). Phosphorylated STAT5 was assessed by Western blotting (**upper panel, A**). Protein loading was verified by blotting with an anti-total STAT5 antibody (**lower panel, A**). **(B)** shows time-course data for the effect of TSLP on pHLF STAT3 phosphorylation. pHLFs were exposed to control medium or TSLP (1 ng/ml) as above. Phosphorylated STAT3 was assessed as above (**upper panel, B**) and protein loading was verified by blotting with an anti-total STAT3 antibody (**lower panel, B**). Data are representative of three independent experiments performed.

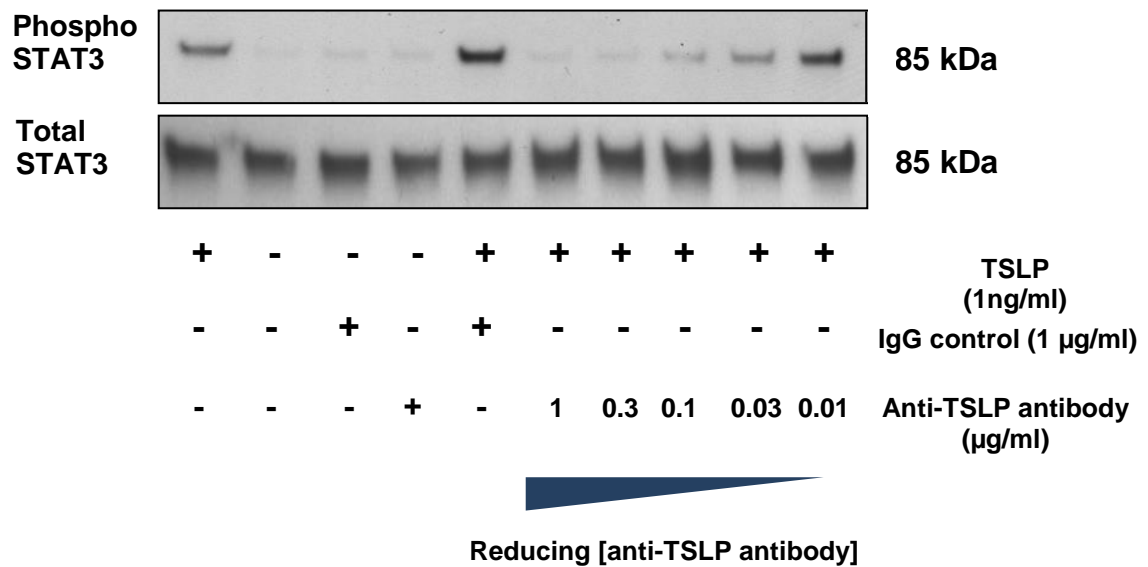


Figure 3.30.

TSLP-induced phosphorylation of STAT3 in pHLFs is inhibited by an anti-TSLP antibody.

Figure shows the effect of an anti-TSLP antibody on TSLP-induced STAT3 phosphorylation in pHLFs at 30 minutes. pHLFs were exposed to control medium; isotype control antibody only (1 µg/ml); anti-TSLP antibody only (1 µg/ml); TSLP (1 ng/ml) pre-incubated with isotype control antibody (1 µg/ml) or varying concentrations of anti-TSLP antibody for 2 hours. Phosphorylation of STAT3 was assessed by Western blotting of cell lysates (**upper panel**). Protein loading was verified by blotting with a total STAT3 antibody (**lower panel**). The blots are representative of three independent experiments performed.

3.3.6. The role of STAT3 in mediating TSLP-induced CCL2 protein release

Having demonstrated that TSLP promotes both CCL2 protein production and STAT3 phosphorylation in pHLFs, I sought to determine if TSLP-induced CCL2 expression in pHLFs was STAT3-dependent. For these studies, I used a specific inhibitor, S3I-201, which has previously been shown to specifically inhibit phosphorylation of STAT3 (Pang *et al.*, 2010)

I first examined the effect of S3I-201 on TSLP-induced STAT3 phosphorylation. Cells were incubated with increasing concentrations of S3I-201 (0 – 300 μ M) prior to exposure to TSLP (1 ng/ml) or control medium. STAT3 phosphorylation was assessed at 30 minutes by Western blotting of cell lysates. **Figure 3.31. (A)** shows that S3I-201 inhibits TSLP-induced STAT3 phosphorylation in a concentration-dependent manner from 10 μ M (-log 5 M) onwards. At concentrations of 100 μ M (-log 4.0 M) onwards, TSLP-induced STAT3 phosphorylation was completely blocked.

Having demonstrated that S3I-201 effectively inhibited TSLP-induced-STAT3 phosphorylation, I next examined the effect of this inhibitor on TSLP-induced CCL2 expression. Cells were exposed to the same experimental conditions as above and CM analysed for CCL2 protein release at 6 hours by ELISA. **Figure 3.31 (B)** shows that S3I-201 inhibits TSLP-induced CCL2 protein release in a concentration dependent manner from 10 μ M (-log 5 M) onwards with complete inhibition of this response observed at a concentration of 300 μ M (-log 3.5 M). The concentrations required to inhibit TSLP-induced CCL2 protein release in the present studies are compatible with previous work (Pang *et al.*, 2010; Siddiquee *et al.*, 2007) as well as the documented IC₅₀ for this inhibitor (86 \pm 33 μ M, Calbiochem, USA). These data therefore suggest that TSLP-induced CCL2 expression in pHLFs is STAT3 dependent.

An inherent issue with using pharmacological inhibitors to investigate signalling pathways is the potential of off-target effects. Therefore, to confirm the importance of STAT3 in mediating TSLP-induced CCL2 expression, I again used a genetic approach. pHLFs were transfected with siRNA targeted against STAT3, scrambled siRNA or simply mock-transfected for 24 hours as described in **Materials and Methods**. Following 24 hours quiescence, cells were exposed to TSLP (1 ng/ml) for 6 hours. Cell lysates were collected and assessed for STAT3 expression by Western blotting. Time-matched CM were analysed for CCL2 protein release by ELISA. As demonstrated in **Figure 3.32. (A-B)**, transfection with STAT3 siRNA resulted in a significant reduction in STAT3 protein expression (~50%) compared to

control cells. This knockdown in STAT3 expression was associated with a significant inhibition (~50%) of TSLP-induced CCL2 protein release (**Figure 3.33**). These data, therefore, lend strong confirmation to the conclusion that TSLP-induced CCL2 release is STAT3 dependent.

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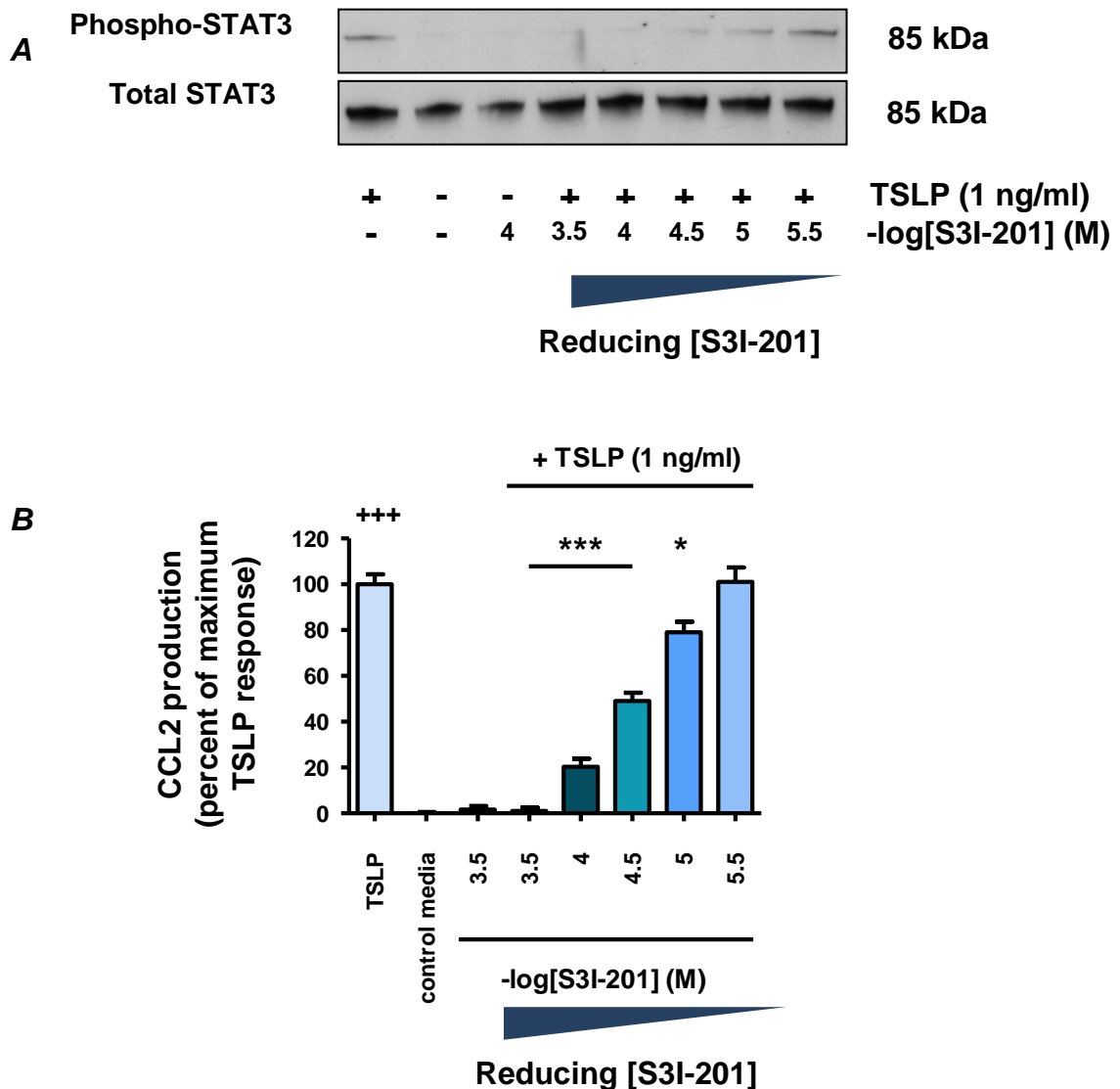


Figure 3.31.

TSLP-induced STAT3 phosphorylation and CCL2 protein release in pHLFs is attenuated in a concentration-dependent manner by the STAT3, S3I-201.

Figure shows the effect of the specific STAT3 inhibitor, S3I-201, on STAT3 phosphorylation (**Panel A**) and CCL2 protein release (**Panel B**) by pHLFs following exposure to TSLP. Cells were treated with increasing concentrations of S3I-201 for 30 minutes prior to exposure to TSLP (1 ng/ml) for 6 hours. Final concentrations of DMSO were kept constant for all experimental conditions (0.1% DMSO in DMEM). Phosphorylation of STAT3 (**upper panel, A**) and protein loading (**lower panel, A**) was assessed by Western blotting as previously described. The blots are representative of three independent experiments performed. **Panel B**: the first bar represents the CCL2 response to TSLP and drug vehicle alone. The second bar represents the response to control medium alone. The third bar represents highest concentration of S3I-201 examined and shows that this compound has no effect on basal CCL2 release. Negative log of the concentrations of S3I-201 are presented. Data represent the mean \pm SEM, from triplicates. * $p < 0.05$; *** $p < 0.001$, comparison with TSLP alone; +++ $p < 0.001$, comparison with untreated cells; one-way ANOVA.

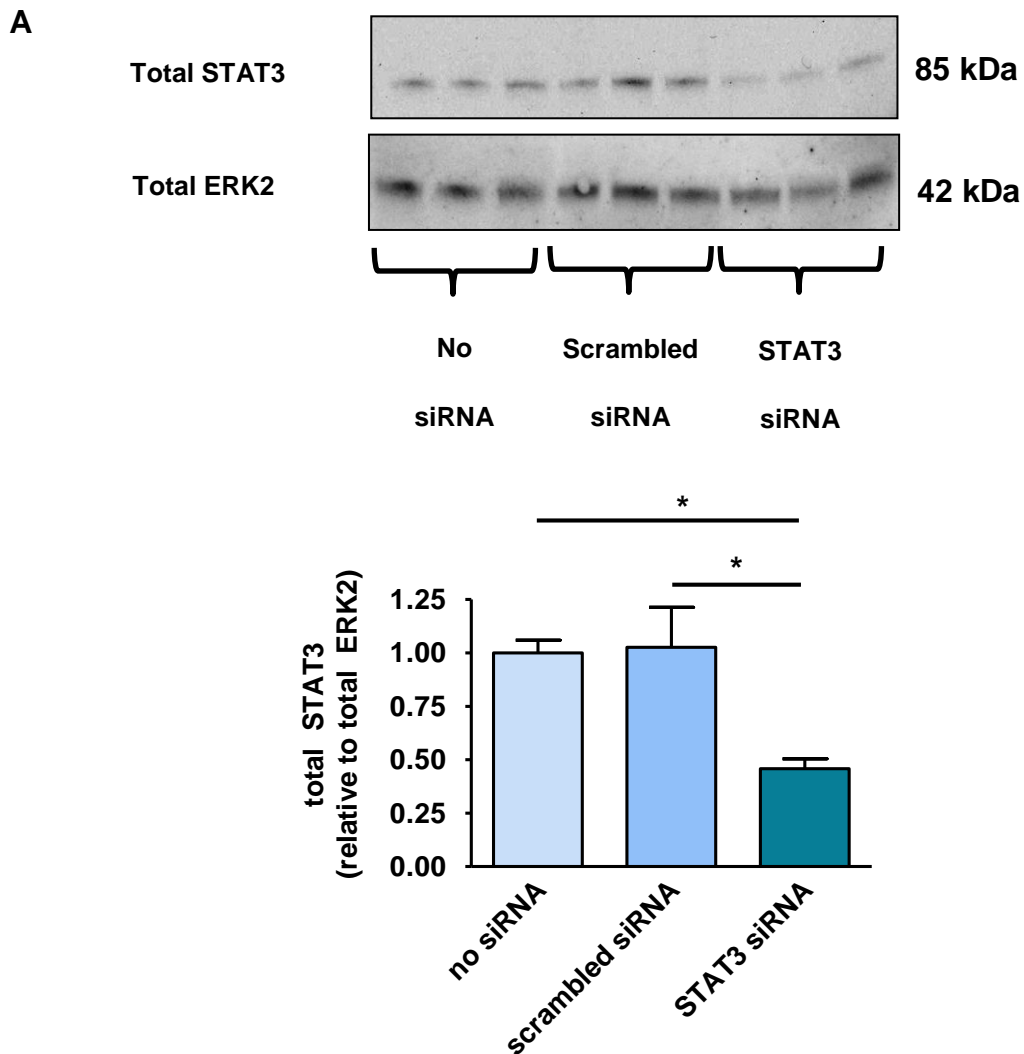


Figure 3.32.

Transfection of pHLFs with siRNA results in successful knock-down of STAT3 protein expression.

Figure shows that transfection of pHLFs with STAT3 specific siRNA results in knockdown of STAT3 expression at the protein level. 48 hours following mock-transfection, or transfection with scrambled or STAT3 specific siRNA (100nM), pHLFs were exposed to TSLP (1 ng/ml) for 6 hours. Expression of STAT3 was assessed by Western blotting of cell lysates (**upper panel, A**); protein loading was confirmed by blotting with an anti-ERK2 antibody (**lower panel, A**). The relative expression of STAT3 normalized to total ERK2 was determined by performing quantitative densitometry (**Panel B**). Data represent the mean \pm SEM from triplicates and are representative of three separate experiments performed. * $p < 0.05$, comparison to mock-transfected cells; one-way ANOVA.

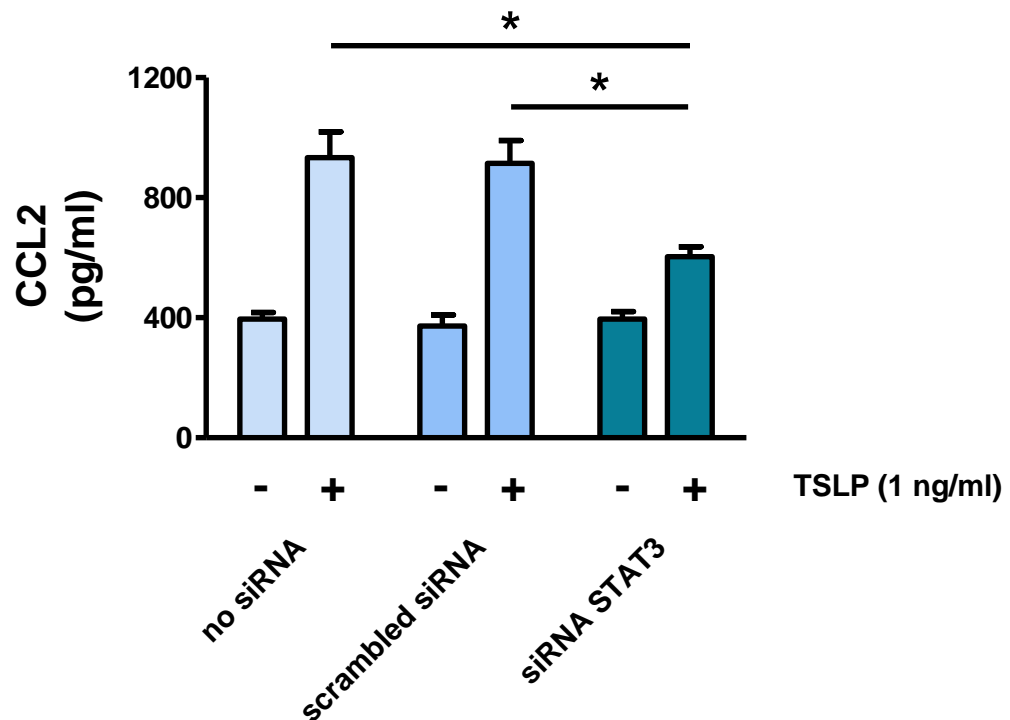


Figure 3.33.

Transfection of pHLFs with siRNA targeted against STAT3 results in an inhibition of TSLP-induced CCL2 protein release.

Figure shows the effect of transfection of pHLFs with STAT3 siRNA on TSLP-induced CCL2 protein release. 48 hours after mock-transfection, or transfection with scrambled siRNA or STAT3-specific siRNA (100 nM), pHLFs were exposed to TSLP (1 ng/ml) or control medium for 6 hours. Expression of STAT3 was assessed by Western blotting as shown in **Figure 3.32**. Time-matched supernatants were collected and analysed for CCL2 protein release by ELISA. The amount of secreted CCL2 is expressed as pg/ml, and each value represents the mean \pm SEM, from triplicates. * $p < 0.05$ comparison with mock-transfected cells; one-way ANOVA.

3.3.7. *The effect of conditioned media of pHLFs exposed to TSLP on human monocyte chemotaxis*

The data presented in the preceding sections demonstrate that TSLP induces upregulation of CCL2 expression in pHLFs. CCL2 is a member of the CC chemokine family and is a potent chemoattractant for inflammatory cells, including mononuclear cells and T cells (Rose *et al.*, 2003). Interestingly, recent evidence has also highlighted the importance of fibroblast-derived CCL2 in mediating dendritic cell trafficking into the lung (Kitamura *et al.*, 2011). The next series of experiments was therefore designed to determine if CCL2 derived from TSLP-treated pHLFs was capable of inducing chemotaxis of human immune cells *in vitro*.

3.3.7.1. *Confirmation of CCL2 induced chemotaxis of human monocytes*

Chemotaxis of the human monocyte / macrophage cell line, THP-1, in response to human recombinant CCL2 (hrCCL2), was assessed using a Boyden chamber as described in **Material and Methods**. Briefly, monocytes were exposed to varying concentrations of hrCCL2 (0 – 100 ng/ml) and chemotaxis assessed by counting cells which had migrated across a 8 µm filter over a 2 hour incubation period using light microscopy. Data are presented as mean number of cells per 5 high powered fields (x40). As can be seen in **Figure 3.34. (A)** hrCCL2 induces significant chemotaxis of THP-1 cells, compared to control medium, in a concentration-dependent manner between 0.1 ng/ml and 3 ng/ml (-log 10 g/ml to -log 8.5 g/l). The overall chemotactic response to hrCCL2 was bell-shaped with optimum chemotaxis (12-fold increase compared to control) induced at a concentration of 3 ng/ml (-log 8.5 g/l).

The specificity of this response for hrCCL2 was examined using a polyclonal goat IgG anti-CCL2 antibody. hrCCL2 (3 ng/ml) was pre-incubated with varying concentrations of anti-hCCL2 antibody (0 – 30 µg/ml) or isotype control (30 µg/ml) for 2 hours at 4°C prior to exposure to THP-1 cells. As can be seen in **Figure 3.34. (B)**, neutralisation of CCL2 resulted in a significant concentration-dependent inhibition of monocyte chemotaxis from 3 µg/ml (-log 5.5 g/l) onwards, with abrogation of chemotaxis observed at an antibody concentration of 30 µg/ml (-log 4.5 g/l). No such inhibition of chemotaxis was observed for cells exposed to hrCCL2 pre-incubated with isotype control. The ND₅₀ of this antibody for this response was determined to be 5.06 µg/ml

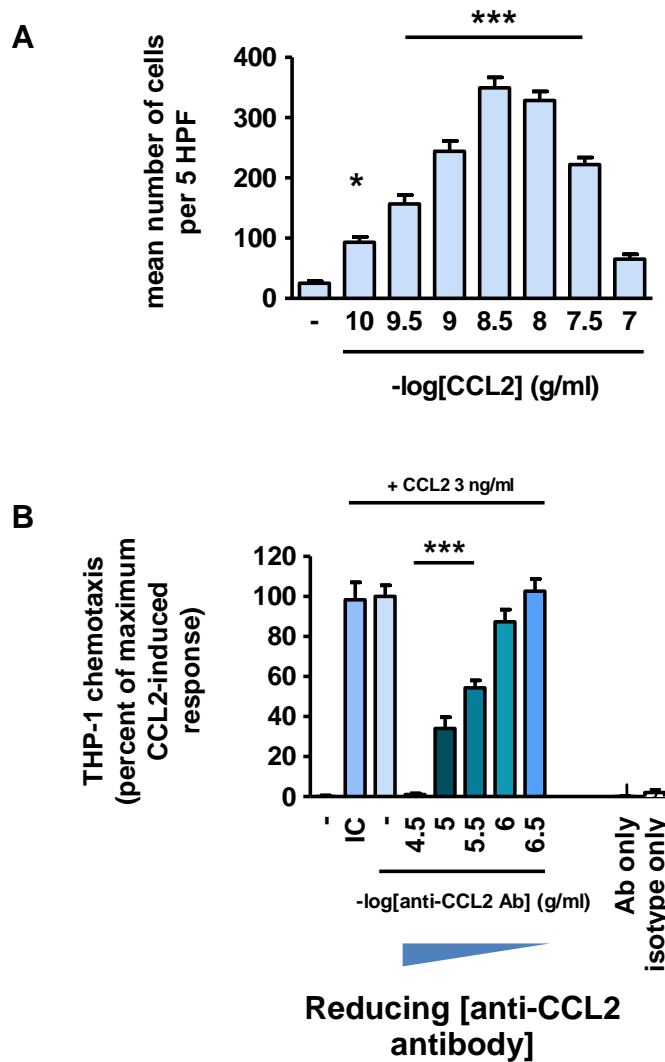


Figure 3.34.

CCL2 promotes chemotaxis of THP-1 mononuclear cells.

Panel A shows the number of THP-1 mononuclear cells (visualized by light microscopy) in high powered fields (HPF) that have migrated towards varying concentrations of recombinant human CCL2 (hrCCL2) over a 2 hour period. Data are presented as the mean number of cells per 5 HPF \pm SEM, from triplicates. * $p < 0.05$; *** $p < 0.001$, comparison with control medium only; one-way ANOVA. **Panel B** shows the effect of varying concentrations (0 – 30 μ g/ml) of anti-CCL2 antibody (Ab) or isotype control Ab (IC) (30 μ g/ml) on hrCCL2-induced THP-1 cell chemotaxis (3 ng/ml). Data are presented as a percentage of the maximal response obtained with hrCCL2 alone. The first bar represents the response to control medium alone. The second bar represents the response to hrCCL2 pre-incubated with the maximum concentration of IC. The final two bars represent the response to maximum concentrations of Ab and IC alone and show that these antibodies had no effect on cell chemotaxis. Data represent the mean \pm SEM, from triplicates; *** $p < 0.001$, comparison with hrCCL2 alone; +++ $p < 0.001$, comparison with control medium alone; one-way ANOVA.

3.3.7.2. *Conditioned media of pHLFs exposed to TSLP induces chemotaxis of human monocytes in a CCL2-dependent manner*

Having confirmed the sensitivity of THP-1 cells to CCL2-induced chemotaxis, I next examined monocyte chemotaxis in response to CM from pHLFs exposed to TSLP (0 – 1 ng/ml) for 6 hours, using the same technique as above. **Figure 3.35. (A)** shows that no significant chemotaxis was observed in response to CM from pHLFs exposed to control media alone or TSLP at concentrations of 0.01- 0.3 ng/ml (-log 11 g/l to -log 9.5 g/l). However, significant monocyte chemotaxis was induced by CM from pHLFs exposed to TSLP concentrations of 1 ng/ml (-log 9 g/l) (5-fold increase over control). This represents a ~65% induction compared with the hrCCL2-induced chemotactic response.

I next assessed whether this response was mediated by CCL2 release into supernatants by pHLFs exposed to TSLP. CM from pHLFs exposed to TSLP (1 ng/ml) for 6 hours was pre-incubated with anti-CCL2 antibody (30 µg/ml) or isotype control antibody (30 µg/ml) as described above, prior to exposure to monocytes. As can be seen from **Figure 3.35. (B)**, monocyte chemotaxis in response to CM from pHLFs exposed to TSLP (1 ng/ml) was significantly attenuated (~40%) in the presence of an anti-CCL2 antibody; no inhibition was observed in the presence of an isotype control antibody. hrTSLP (1 ng/ml) exerted no chemotactic effect on THP-1 cells demonstrating that the observed chemotactic response was not due to direct stimulation by TSLP. Interestingly, neutralisation of CCL2 did not result in a complete inhibition of monocyte chemotaxis induced by CM from pHLFs exposed to TSLP, suggesting TSLP may induce the generation of other chemotactic agents by pHLFs.

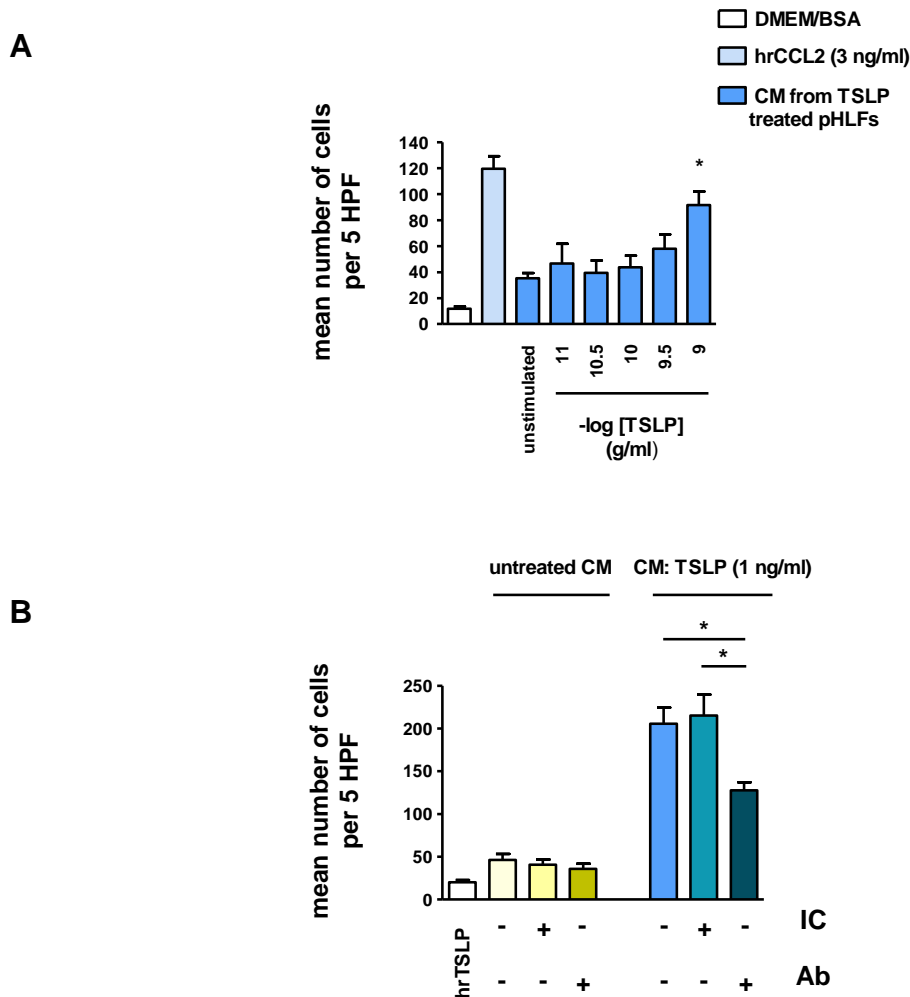


Figure 3.35.

Conditioned media from TSLP-treated pHLFs induces chemotaxis of THP-1 monocytes via CCL2.

Panel (A) shows the effect on chemotaxis of THP-1 monocytes induced by conditioned media collected from pHLFs exposed to control medium or varying concentrations of TSLP for 6 hours. The first bar represents the chemotactic response of cells to DMEM/BSA (1%, w/v) only. The second bar represents the chemotactic response of cells to hrCCL2 (3 ng/ml). Data are presented as the mean number of cells per 5 HPF \pm SEM, from triplicates. * $p < 0.05$, comparison with conditioned media from unstimulated cells; one-way ANOVA. **Panel (B)** shows the effect of an anti-CCL2 antibody (Ab) or isotype control (IC) (both at 30 $\mu\text{g/ml}$) on chemotaxis of THP-1 cells induced by conditioned media from pHLFs exposed to control medium or TSLP (1 ng/ml) for 6 hours. The first bar represents the chemotactic response of cells to human recombinant (hr) TSLP (1 ng/ml). Data are presented as **Panel A**. * $p < 0.05$, comparison with conditioned media from TSLP-treated pHLFs not incubated with Ab or IC; one-way ANOVA.

3.3.8. Summary

The results described in this section, examining the responsiveness of primary human lung fibroblasts (pHLFs) to TSLP, shows that:

- pHLFs express both components of the TSLP receptor;
- TSLP induces STAT3, but not STAT5 phosphorylation in pHLFs;
- TSLP upregulates the expression of CCL2 in pHLFs *in vitro* in a time-dependent manner;
- STAT3 phosphorylation, subsequent to TSLPR ligation, mediates CCL2 expression in pHLFs;
- TSLP induces chemotaxis of human monocytes *in vitro* in a CCL2-dependent manner

Taken together, these data demonstrate for the first time that primary human lung fibroblasts express a functional TSLP-TSLP receptor signalling axis and that this leads to the upregulation of CCL2 release, an important immune-modulatory chemokine. Moreover, they highlight a novel role for TSLP in the generation of a fibroblast-derived chemokine gradient.

3.4. The role of TSLP in the development of Type-2 immune responses in the lung following non-allergen mediated lung injury

3.4.1. Introduction

The data presented so far in this thesis has demonstrated that lung fibroblasts represent both a potential cellular source and target of TSLP, an important T-2 polarising cytokine, in IPF, a condition characterised by increased expression of pro-fibrotic T-2 cytokines (Wallace *et al.*, 1995). The strong immunoreactivity for TSLP and TSLPR in IPF lung suggests a potential pathogenetic role for this cytokine in the development of lung fibrosis, and the next series of experiments were designed to explore this concept in an animal model of bleomycin-induced lung injury and fibrosis. This model cannot claim to faithfully recapitulate all the features of IPF. Nonetheless, it does share some important features which have allowed the identification of important pro-fibrotic pathways that have relevance in human disease. In particular, the development of inflammatory / immune responses in this model, and the subsequent development of fibrosis, do not seem dependent on allergen exposure, but rather follow epithelial injury. In the first instance, I examined the time-course of TSLP immunoreactivity in murine lung following bleomycin challenge over 28 days. I also examined TSLPR immunoreactivity during the fibrotic phase of this model. Thereafter, I performed FACS analysis on murine lung single cell suspensions following bleomycin challenge to profile local immune cell populations following lung injury in this model. In addition, concomitant studies were performed to determine the role of TSLP in the development of bleomycin-induced immune responses. Finally, the contribution of TSLP to the development of bleomycin-induced lung fibrosis, as assessed by lung collagen deposition, was examined. For these studies, bleomycin (2 mg/kg body weight) or saline was administered via the oropharyngeal route to C57Bl/6 mice as described in **Materials and Methods**. To evaluate the role of TSLP in these responses, a rat monoclonal IgG neutralising anti-TSLP antibody (28F12), or isotype control (IC) was administered via the intra-peritoneal (i.p.) route at a dose of 15 mg/kg, every 4 days. These antibodies were kind gifts from GlaxoSmithKline (GSK, UK). This dosing schedule was chosen based on previous pharmacokinetic data made available by GSK, and a prophylactic regime was employed, with antibody intervention being initiated on day -1. Appropriate specificity and affinity of these antibodies had previously been confirmed on a Biacore Systems platform by GSK. The efficacy of 28F12 to neutralise TSLP activity was further confirmed by demonstrating a concentration-dependent inhibition of TSLP-induced CCL2 protein release in primary murine lung fibroblasts (pMLFs) *in vitro* (please see **Appendix A2**). Serum

samples were taken from mice used in intervention studies and anti-TSLP and isotype control antibody levels were measured by ELISA at GSK.

3.4.2. Immunohistochemical localisation of TSLP and TSLPR in bleomycin induced lung injury and fibrosis

Having demonstrated significant TSLP immunoreactivity for injured hyperplastic epithelial cells and fibroblasts in IPF lung, I examined the cellular localisation of TSLP and TSLPR in the lungs of mice challenged with bleomycin over time to determine if a similar pattern was apparent in experimentally-induced fibrosis. Mice challenged with bleomycin (2 mg/kg) or saline were sacrificed at days 3, 7, 14, 21 and 28. Sections were prepared as described in **Materials and Methods** and examined by immunohistochemistry using non-biotinylated polyclonal rabbit anti-TSLP and anti-TSLPR antibodies. Antigen retrieval and optimal concentrations of the primary antibodies used for these studies are outlined in **Table 2.1**.

Figures 3.36-3.38 show the immunohistochemical localisation of TSLP in control lung and in the bleomycin model of lung injury and fibrosis, respectively. Tissue sections from saline-treated animals (**Figure 3.36**) demonstrated predominantly weak staining throughout which was associated with alveolar epithelial cells, and macrophages. Occasional strongly positive alveolar epithelial cells and macrophages were observed, though there was no discernible geographical or temporal pattern to this. This pattern was observed at all time points following saline treatment. A similar pattern of immunoreactivity was observed in the lungs of mice harvested 3 days post bleomycin-challenge (**Figure 3.37 Panel A**). However, from day 7 onwards (**Figure 3.37 Panels B-E**), strong TSLP immunoreactivity was observed for cells with the morphological characteristics of fibroblasts, as well as alveolar epithelial cells and macrophages. Alveolar epithelial cell immunoreactivity was more pronounced in areas of increased matrix deposition. This pattern was maintained at all time points evaluated to day 28 post bleomycin-challenge. **Figure 3.38. (Panel A)** shows **Figure 3.36 Panel (C)** at a higher magnification (X20). Spindle-shaped fibroblasts within areas of peri-bronchiolar matrix deposition can be observed to be strongly immunoreactive for TSLP, together with alveolar and bronchial epithelial cells. No discernible staining was observed on serial sections stained with isotype-specific non-immune primary antibody or primary antibody pre-incubated with a TSLP blocking peptide (**Panels B-C**), as described in **Materials and Methods**, confirming specificity of the immunoreactive signal for TSLP.

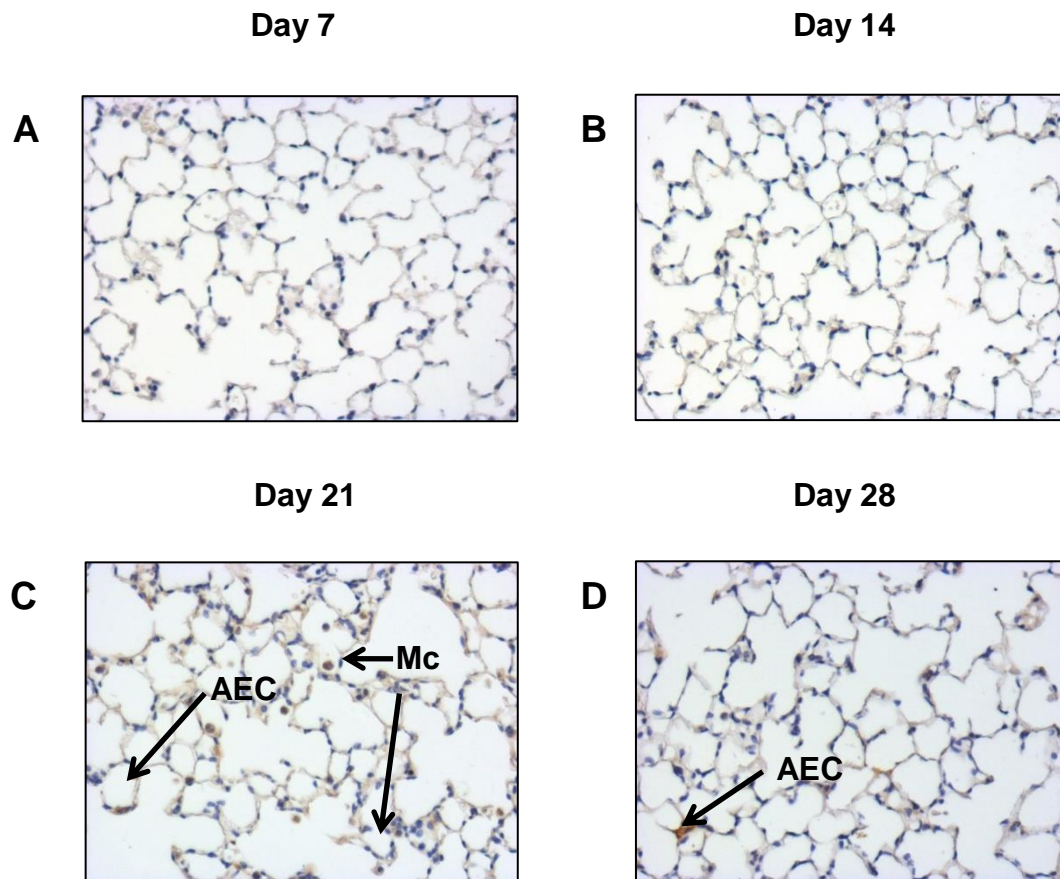


Figure 3.36.

Murine lung TSLP immunostaining in saline-treated mice.

Shown is immunostaining for TSLP in mouse lung 7 (A), 14 (B), 21 (C) and 28 (D) days after saline treatment. In contrast to mice challenged with bleomycin (see Figure 3.37), immunoreactivity for TSLP is less extensive and is observed for macrophages (Mc) and occasional alveolar epithelial cells (AEC). Specific staining is depicted in brown; nuclei are stained blue with haematoxylin. n=3 for all groups; sections are representative for all mice; original magnification x20. For staining with isotype-specific non-immune primary antibody and anti-TSLP antibody pre-incubated with blocking peptide, please see Figure 3.37.

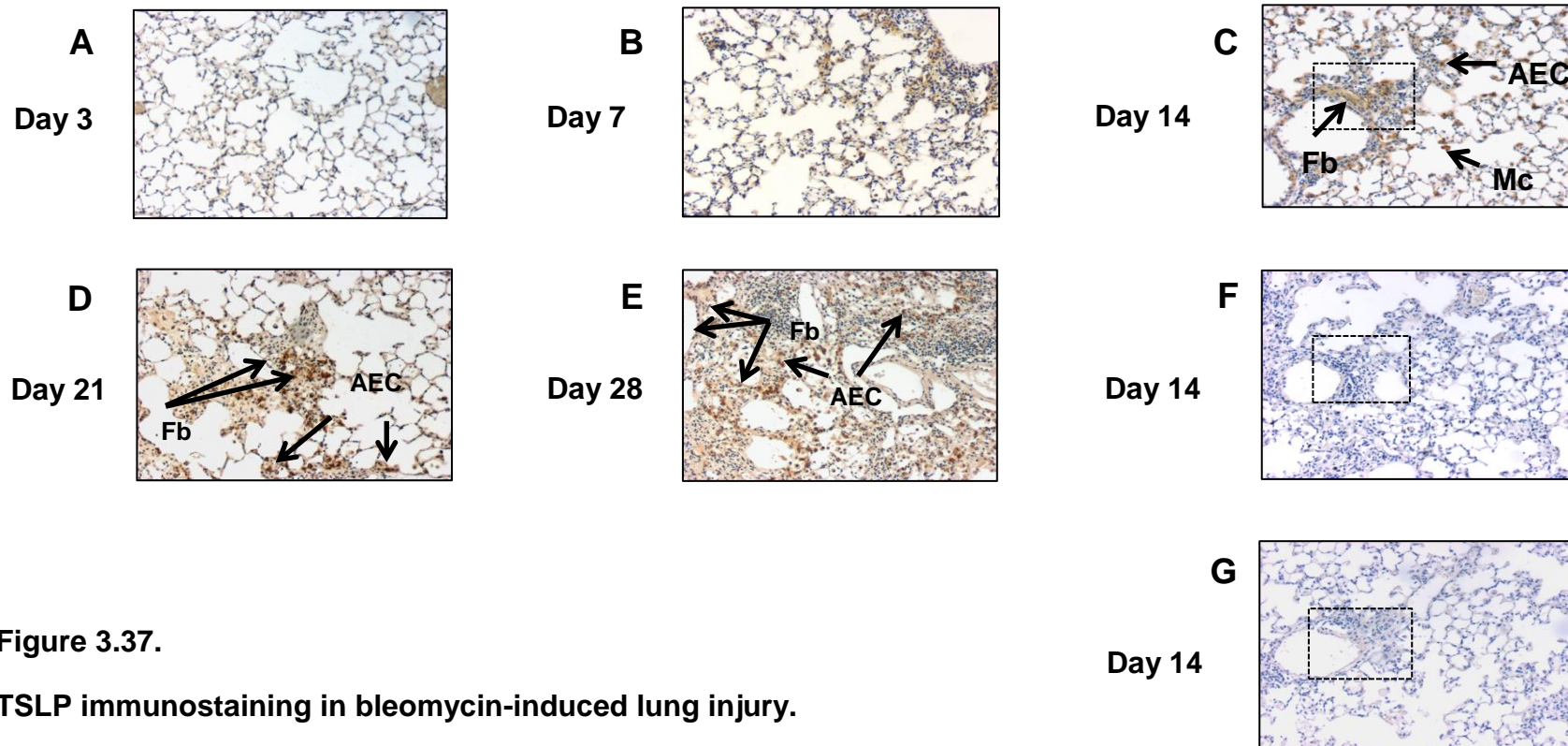


Figure 3.37.

TSLP immunostaining in bleomycin-induced lung injury.

Shown is immunolocalisation of TSLP (**A-E**) in lung sections 3 (**A**), 7 (**B**), 14 (**C**), 21 (**D**), and 28 (**E**) days after oropharyngeal challenge with bleomycin (2 mg/kg). Murine lung TSLP immunoreactivity is weak/absent at day 3 post bleomycin-challenge (**A**). In contrast, strong immunoreactivity for TSLP is observed on cells with the morphology of fibroblasts (Fb), alveolar epithelial cells (AEC) and macrophages (Mc) from day 7 onwards (**B, C, D, and E**). Fibroblast immunoreactivity is more apparent in areas of increased matrix deposition, both peri-bronchiolar (**C**) and interstitial (**D-E**) which becomes more apparent with time post bleomycin challenge. No discernible staining for TSLP is observed on tissue sections, serial to (**C**) from bleomycin-challenged mice stained with isotype-specific non-immune primary antibody (**F**) or with anti-TSLP antibody pre-incubated with blocking peptide (1:6) (**G**). $n=3$ for all time points; sections shown are representative of all mice observed. Original magnifications x10. Increased magnification (x20) of section (**C, F and G**) are represented in **Figure 3.38**.(dotted area).

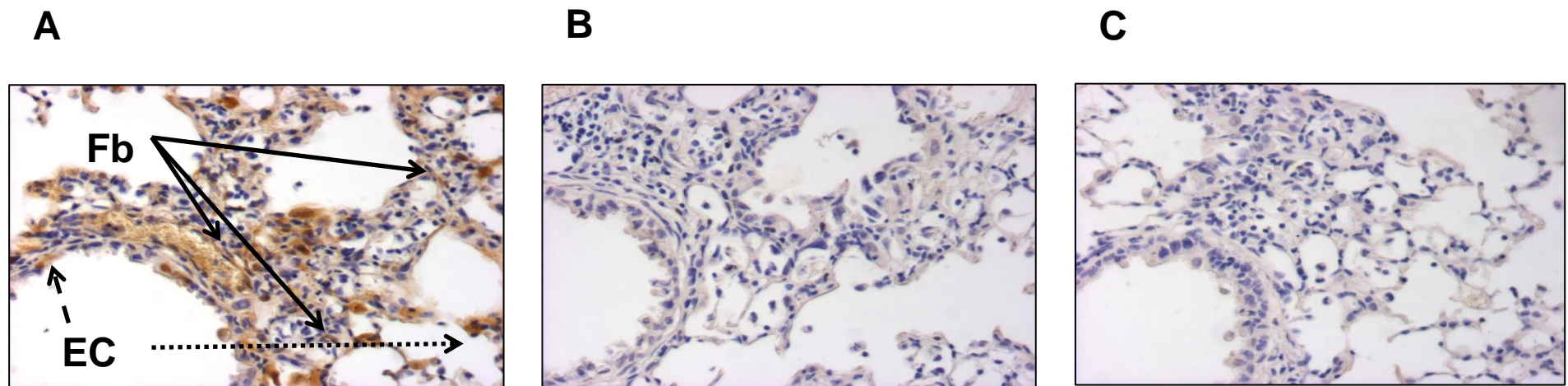


Figure 3.38.

TSLP immunostaining in bleomycin-induced lung injury (2).

Shown is immunolocalisation of TSLP **(A)** 14 days after oropharyngeal challenge with bleomycin (2 mg/kg). Strong immunoreactivity for TSLP is observed in fibroblasts (Fb) within areas of increased matrix deposition. TSLP immunoreactivity is also observed for epithelial cells (EC), both bronchial (dashed arrows) and alveolar (dotted arrows). No discernible staining is observed on serial tissue sections stained with isotype-specific non-immune primary antibody **(B)**. Loss of signal is also observed in serial sections stained with primary anti-TSLP antibody, pre-incubated with blocking peptide (1:6) **(C)**. $n=3$ for bleomycin-challenged mice; sections shown are representative of all mice. Original magnification x20.

Figure 3.39. shows the immunohistochemical localisation of TSLPR in murine fibrotic lung at day 14 post-bleomycin challenge. Tissue sections from saline-instilled animals demonstrated positive staining on alveolar epithelial cells (**Panel A**). However, a number of these cells also displayed small cytoplasmic stellate projections characteristic of immature DCs. Tissue sections from bleomycin-challenged mice (**Panels B-D**) showed intense staining for TSLPR in areas of fibrosis, localising to bronchial epithelial cells, alveolar epithelial cells and fibroblasts. In addition, cells with the morphological characteristics of DCs, located within aggregates of mononuclear cells, were also observed to be strongly positive for TSLPR. These aggregates were typically organised in a bronchocentric pattern. Again, no discernible staining was observed for TSLPR on serial sections stained with isotype-specific non-immune primary antibody, demonstrating specificity of the immunoreactive signal for TSLPR (**Panel E**).

These data therefore suggest that the immunolocalisation of TSLP might be similar in both experimentally-induced fibrosis and IPF, identifying epithelial cells and fibroblasts as potential cellular sources of this cytokine in lung fibrogenesis. Moreover, the increase in signal strength from day 3 onwards suggests a time-dependent increase in TSLP expression following bleomycin-induced lung injury. In addition, these studies demonstrate that, in addition to immune cells organised within lymphoid aggregates in fibrotic lung, structural cells, including epithelial cells and fibroblasts, may be capable of responding to TSLP. These murine findings are consistent with the data presented in **Section 3.3.** demonstrating that pHLFs express a functional TSLP-TSLPR signalling axis.

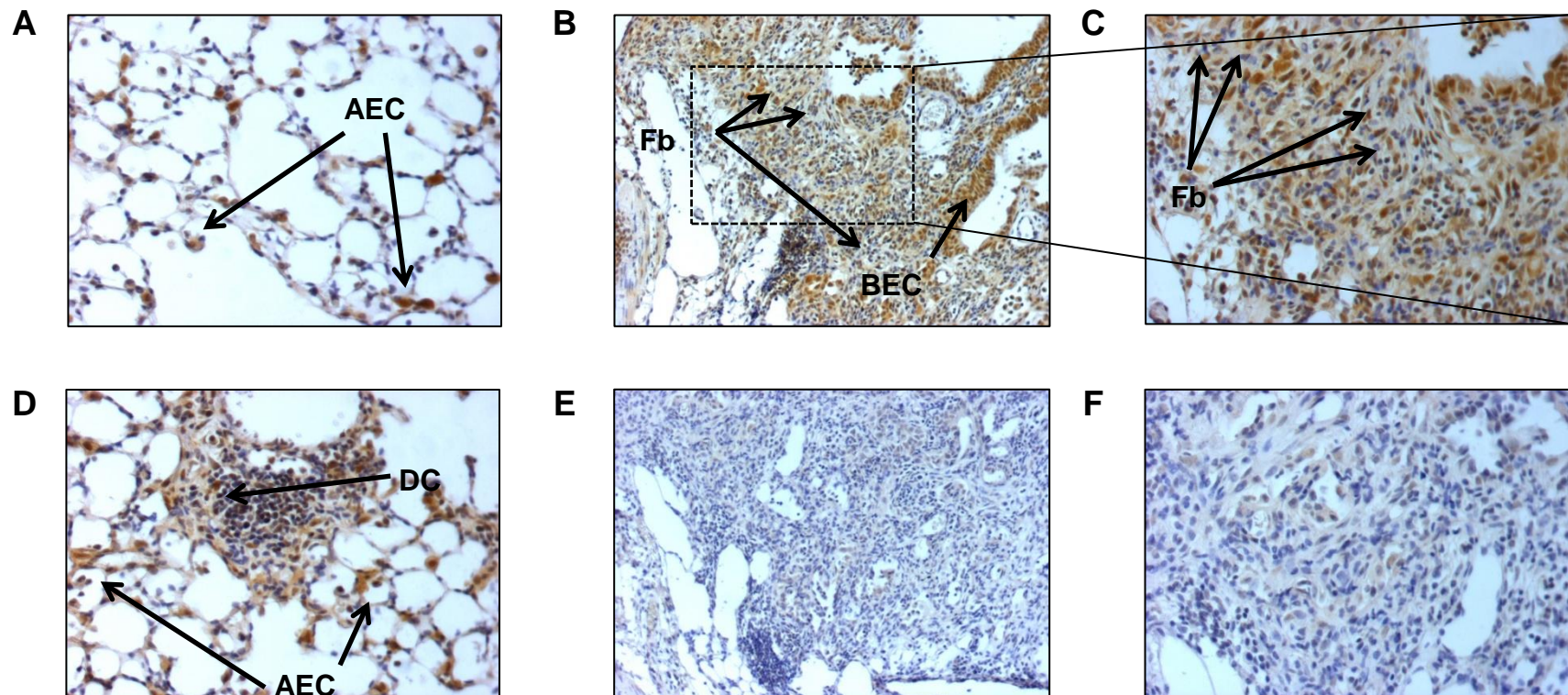


Figure 3.39.

Immunohistochemical localization of TSLPR in bleomycin-induced lung injury and fibrosis.

Shown is immunolocalization of TSLPR (**A-D**) in lung sections 14 days after saline administration (**A**) or bleomycin challenge (**B-D**). In saline-treated control sections (**A**), TSLPR immunoreactivity is observed for alveolar epithelial cells (AEC). In lung sections from mice challenged with bleomycin (**B-D**), TSLPR immunoreactivity is observed for bronchial epithelial cells (BEC), fibroblasts (Fb), alveolar epithelial cells. In addition, strong TSLPR immunoreactivity is observed for cells with the characteristic shape of dendritic cells (DC) within lymphoid aggregates in bleomycin-challenged mice (**D**). Serial sections of (**B**) were stained with isotype-specific non-immune primary antibody (**E, F**), and show no discernible immunoreactivity. Original magnifications x20 (**A, C, D, F**); x10 (**B, E**). n=3 for all groups; sections shown are representative of all mice observed.

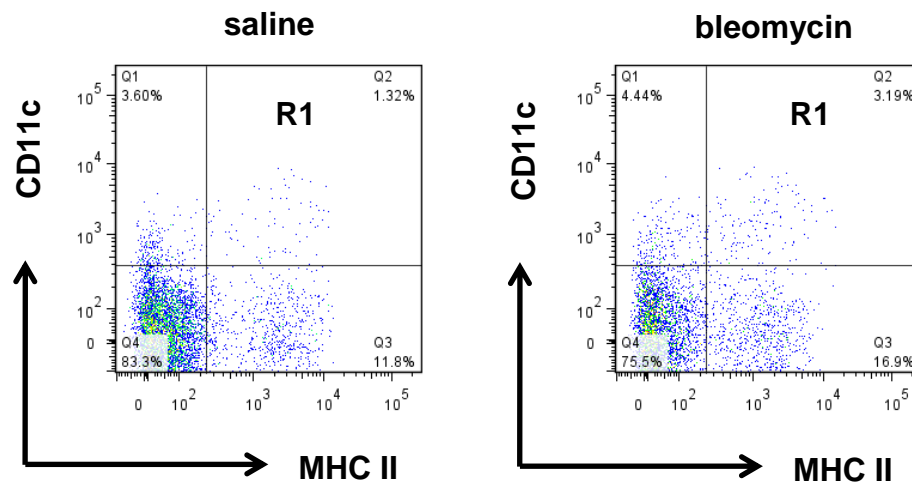
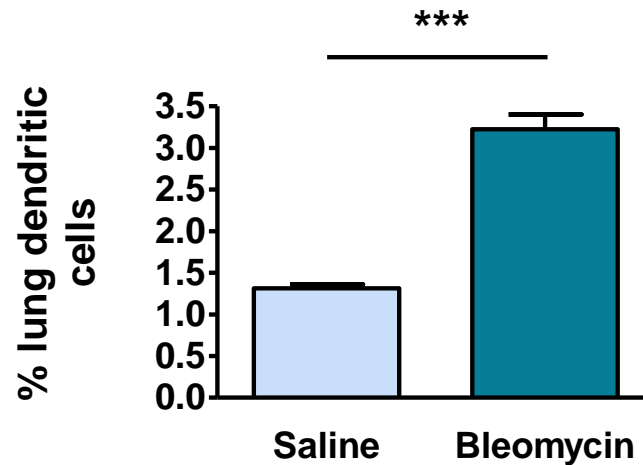
3.4.3. *The effect of bleomycin-induced lung injury on the lung dendritic cell accumulation at day 7*

As discussed in **Sections 1.46-1.47** key cellular targets for TSLP in the development of T-2 immune responses include dendritic cells (DCs) and T-lymphocytes. Having demonstrated a time-dependent increase in TSLP immunoreactivity in murine lung following bleomycin-induced lung injury, I next examined the effect of such injury on lung DC accumulation during the inflammatory phase of this model. Mice challenged with bleomycin or saline were sacrificed at day 7 and lung harvested for the generation of single cell suspensions for FACS analysis, as described in **Materials and Methods**. This time point was chosen on the basis of previous experience with this model, suggesting the development of a lymphocytic alveolitis during the first 7 – 10 days (Janick-Buckner *et al.*, 1989). The development of lung fibrosis, in contrast, is observed by day 14. The gating strategy employed to identify DCs in lung single cells suspensions is described in detail in **Materials and Methods**. Briefly, however, cells were initially gated according to forward and side scatter characteristics to exclude debris and lymphocytes. Data are presented as the percentage of these cells which are DCs. DCs were identified as being CD11c⁺/MHC II^{hi} cells with low autofluorescence, thus excluding alveolar and interstitial macrophages from analysis.

As can be seen from **Figure 3.40.**, bleomycin challenge resulted in a significant increase in the lung DC population (3.2 % \pm 0.14) compared to saline-instilled controls (< 1.5%) at day 7 post challenge. These results demonstrate that bleomycin-induced lung injury is associated with an accumulation of DCs in the lung during the first week post-challenge.

3.4.4. *The role of TSLP in influencing the lung dendritic cell population following bleomycin-induced lung injury at day 10 and 28*

Having demonstrated that bleomycin challenge results in an accumulation of dendritic cells during the inflammatory phase of this model, I next examined if this response was maintained at later time points; furthermore, by neutralising TSLP activity *in vivo*, I examined the potential role of TSLP in mediating DC recruitment to the lung following bleomycin-induced lung injury. For this series of experiments, mice were challenged with bleomycin or saline, and anti-TSLP antibody (28F12) or IC antibodies administered as described in **Section 3.4.1**. Mice were sacrificed at day 10 and day 28 post-challenge, and FACS analysis performed on single cell suspensions.

A**B****Figure 3.40.**

Bleomycin-induced lung injury leads to an increase in the lung dendritic cell population at day 7 post injury.

Figure shows the effect of bleomycin challenge on lung dendritic cell accumulation at day 7 post-injury. C57Bl/6 mice were instilled with saline or bleomycin (2 mg/kg) via the oropharyngeal (o.p.) route; lungs were harvested from mice for preparation of single cell lung suspensions as per **Materials and Methods**. **Panel (A)**: Representative examples of fluorescence-activated cell-sorting (FACS) dot plots obtained from lung single cell suspensions. CD11c⁺/MHC II^{hi} cells of low autofluorescence were identified as dendritic cells (DCs) – R1. **Panel (B)**: Percentage of dendritic cells (DCs) in lung homogenates of saline-treated mice or bleomycin-challenged mice at day 7 (R1). saline, n=3; bleomycin, n=4; data are presented as mean percentage of lung cells of low autofluorescence which are CD11c⁺/MHC II^{hi} ± SEM; ***p<0.001 Student's paired t-test.

3.4.4.1. *Effect of 28F12 administration on bleomycin-induced lung dendritic cell accumulation*

As can be seen in **Figure 3.41 (A-B)**., the increase in lung DC population following bleomycin challenge, compared to saline control, at day 7 was maintained at day 10 ($5.4\% \pm 0.2$ vs 1.1 ± 0.2). In fact, the percentage of DCs at this later time point in the BLM / IC group was greater than the percentage observed in the BLM group of the earlier experiment ($5.4\% \pm 0.2$ vs $3.2\% \pm 0.12$). These data therefore potentially suggest bleomycin induces lung DC accumulation in a time-dependent manner. The percentage of lung DCs in both groups of saline-instilled mice remained consistently less than 1.5%. The passive neutralisation of TSLP had no effect on bleomycin-induced lung DC accumulation at this time point ($5.4\% \pm 0.2$ vs $5.6\% \pm 2.1$), suggesting that TSLP does not play a role in promoting dendritic cell accumulation to the lung following BLM-induced injury.

Previous studies have suggested that peak expression of T-2 cytokines following bleomycin-induced fibrosis occurs between days 21 and 28. Moreover, a therapeutic benefit in attenuating lung fibrosis has been demonstrated in this model by targeting IL-13 responsive cells during the fibrotic phase of this model (Jakubzick *et al.*, 2003). As shown in **Section 3.4.2.**, immunoreactivity for TSLP remains strong during this phase of injury, suggesting potential activation of DCs into a T-2 polarising phenotype during this time. As can be seen from **Figure 3.42 (A-B)**, at this later time point, bleomycin challenge continued to be associated with an increase in the lung DC population, compared to saline control ($10.6\% \pm 1.1$ vs $5.9\% \pm 0.45$). These data therefore demonstrate that lung DC accumulation increases following bleomycin challenge between days 10 and 28 ($5.4\% \pm 0.2$ vs $10.6\% \pm 0.2$). Interestingly, at this later time point, the percentage of DCs in the lungs of saline-instilled mice also increased to $5.9\% \pm 0.45$, suggesting a baseline recruitment of DCs during the course of these studies. However, as observed at day 10, the neutralisation of TSLP activity *in vivo* had no effect on the bleomycin-induced increase in lung DCs, suggesting that these cells are recruited to the lung in a TSLP-independent manner.

3.4.4.2. *Effect of 28F12 administration on bleomycin-induced lung dendritic cell activation*

Having demonstrated that bleomycin-induced lung injury is associated with an increase in the lung DC population that continues into the fibrotic phase, I sought to characterise the DC phenotype associated with bleomycin challenge further by determining their activation status, as assessed by CD86 expression.

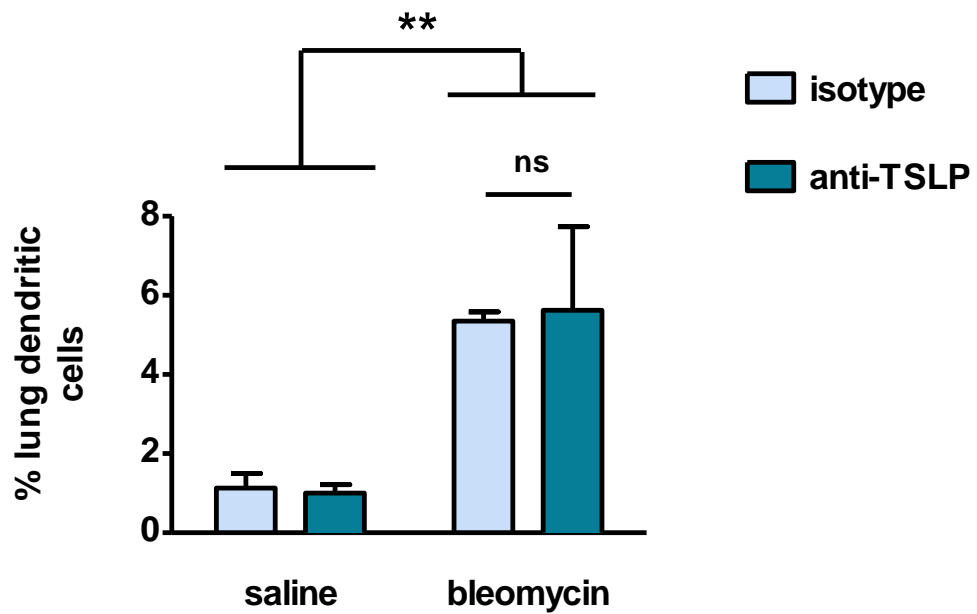


Figure 3.41A.

The bleomycin-induced increase in lung DCs at day 10 is not affected by administration of anti-TSLP antibody.

Figure shows the effect of anti-TSLP antibody administration on bleomycin-induced lung DC accumulation at day 10 post-challenge. C57Bl/6 mice were instilled with saline or bleomycin, and administered isotype control or anti-TSLP antibody i.p. as per **Materials and Methods**. Lungs were harvested for preparation of single cell lung suspensions and gating for DCs was performed as described in text. Data are presented as percentage of lung cells of low autofluorescence which are $CD11c^+/MHC\ II^{hi} \pm SEM$; $n=3$ in all groups; *** $p<0.001$, comparison with saline; ns – non significant, comparison with bleomycin / isotype; two-way ANOVA.

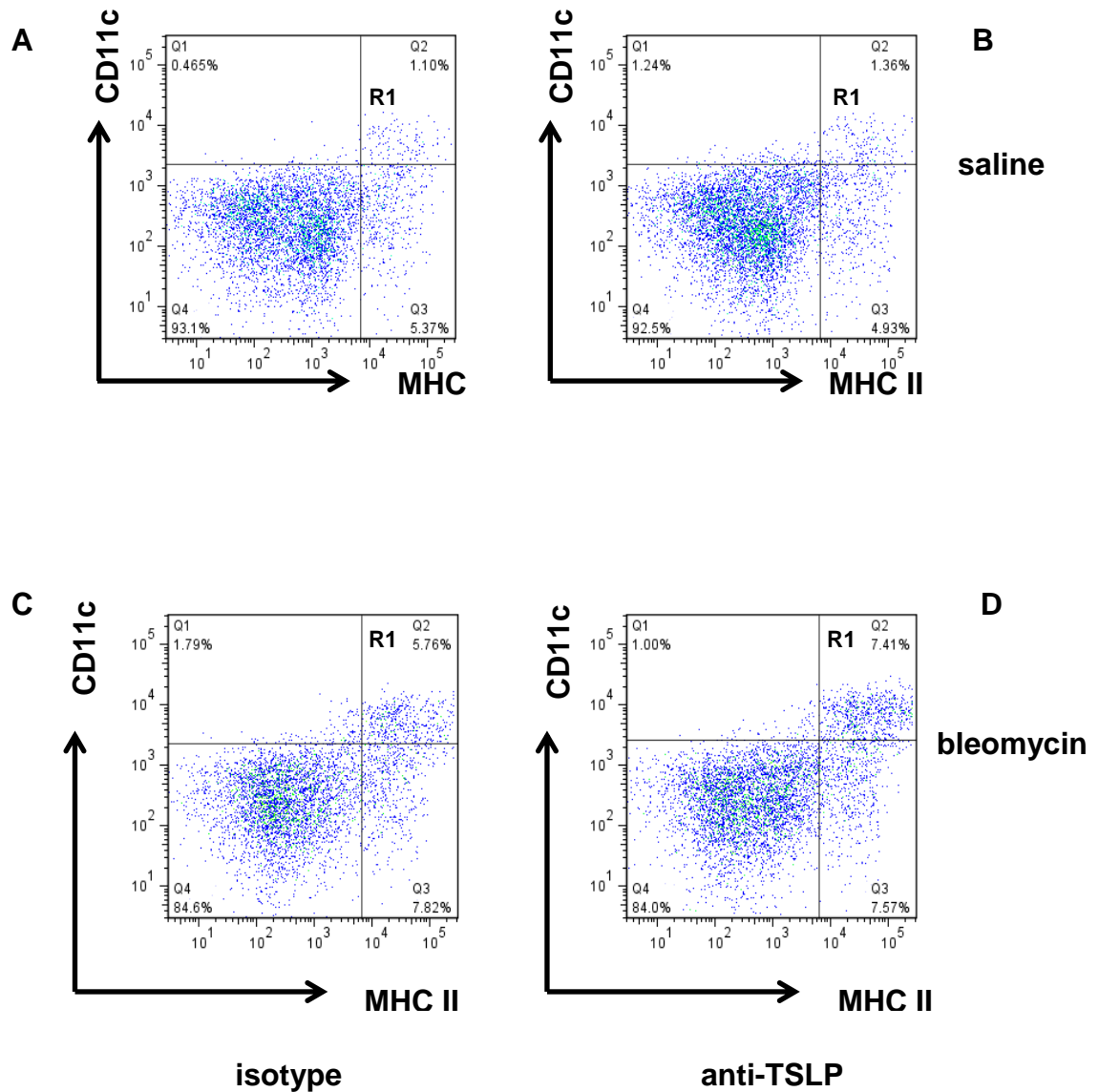


Figure 3.41B.

The bleomycin-induced increase in lung DCs at day 10 is not affected by administration of anti-TSLP antibody.

Figure shows representative FACS plots demonstrating the effect anti-TSLP administration on bleomycin-induced lung DC accumulation at day 10. C57Bl/6 mice were instilled with saline or bleomycin, and administered isotype control or anti-TSLP antibody i.p. as per **Materials and Methods**. Lungs were harvested for preparation of single cell lung suspensions as described in **Materials and Methods**. CD11c⁺/MHC II^{hi} cells of low autofluorescence were identified as dendritic cells (DCs) – R1. **(A)** saline and isotype control antibody; **(B)** saline and anti-TSLP antibody; **(C)** bleomycin and isotype control antibody; **(D)** bleomycin and anti-TSLP-antibody; n=3, all groups.

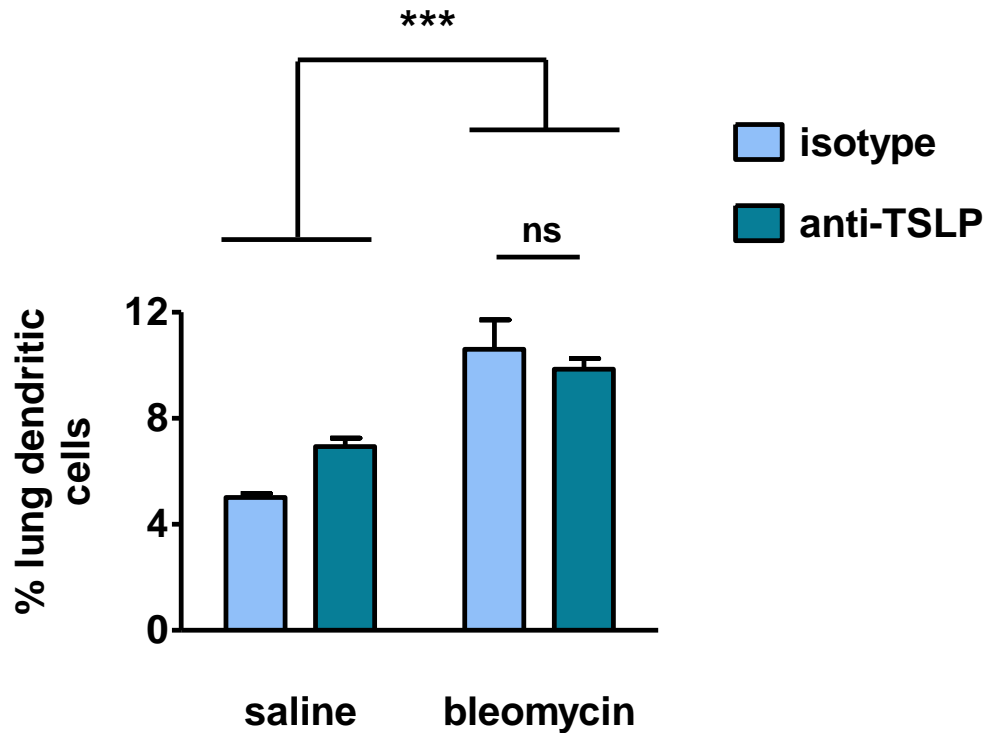


Figure 3.42A.

The bleomycin-induced increase in lung DCs at day 28 is not affected by administration of anti-TSLP antibody.

Figure shows the effect of anti-TSLP administration on bleomycin-induced lung DC accumulation at day 28 post challenge. C57Bl/6 mice were instilled with saline or bleomycin, and administered isotype control or anti-TSLP antibody i.p. as per **Materials and Methods**. Lungs were harvested for preparation of single cell lung suspensions and gating for DCs was performed as described in text. Data are presented as percentage of lung cells of low autofluorescence which are CD11c⁺/MHC II^{hi} \pm SEM; n=3 in all groups; *** p<0.001, comparison with saline; ns – non significant, comparison with bleomycin / isotype; two-way ANOVA.

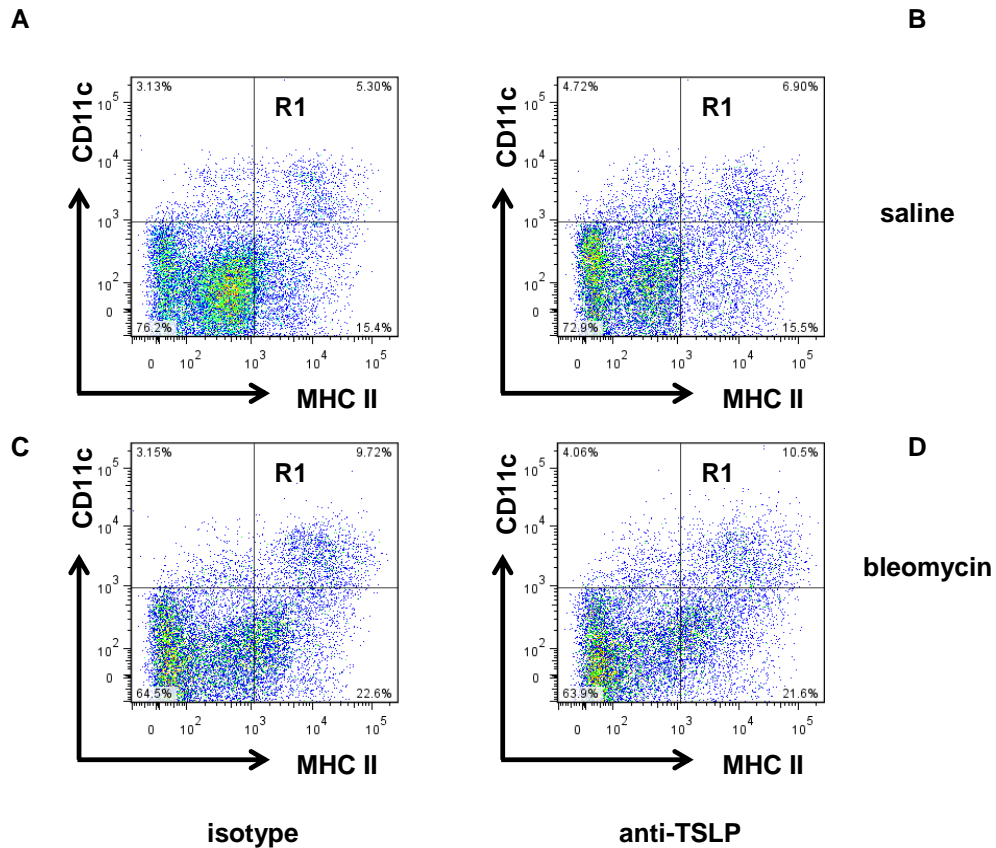


Figure 3.42B.

The bleomycin-induced increase in lung DCs at day 28 is not affected by administration of anti-TSLP antibody.

Figure shows representative FACS plots demonstrating the effect anti-TSLP administration on bleomycin-induced lung DC accumulation at day 28. C57Bl/6 mice were instilled with saline or bleomycin, and administered isotype control or anti-TSLP antibody i.p. as per **Materials and Methods**. Lungs were harvested at 28 days for preparation of single cell lung suspensions as described in **Materials and Methods**. CD11c⁺/MHC II^{hi} cells of low autofluorescence were identified as dendritic cells (DCs) – R1. **(A)** saline and isotype control antibody; **(B)** saline and anti-TSLP antibody; **(C)** bleomycin and isotype control antibody; **(D)** bleomycin and anti-TSLP-antibody; n=3, all groups.

Maturation of DCs permit functional interactions with effector immune cells, including T-cells, and TSLP-activated DCs in particular promote the differentiation of naïve CD4⁺ T-cell into Th2 cells, capable of generating T-2 cytokines. As shown in **Figure 3.43 (A-B)**, bleomycin challenge is associated with a significantly greater proportion of DCs which express CD86, compared with saline controls at day 10 post injury ($26.2\% \pm 0.9$ vs $15.5\% \pm 3.4$). Administration of 28F12 to mice challenged with bleomycin resulted in a significant reduction in the proportion of CD86⁺ DCs ($26.2\% \pm 0.9$ vs $9.8\% \pm 2.2$). No significant differences were observed in the percentage of CD86⁺ lung DCs in the two saline groups. These findings strongly suggest that bleomycin-induced lung injury is associated with DC activation which in turn is TSLP-dependent. No changes were observed in the activation status of splenic DCs following bleomycin challenge compared to saline control (please see **Appendix A3**), suggesting that the maturation of DCs following bleomycin-induced lung injury was limited to the site of injury.

Having previously demonstrated that the lung DC population continues to increase into the fibrotic phase of the bleomycin model (**Section 3.4.4.1.**), I next examined the DC phenotype at this later time point to determine if they remained in an activated state. As well as assessing CD86 expression at this time-point, I also examined OX40L expression by DCs. Quiescent DCs do not express OX40L, but as discussed in **Section 1.4.6.**, TSLP induces upregulation of OX40L expression by DCs; subsequent OX40L-OX40 interactions between TSLP-DCs and T-cells are key to the development of Th2 cells. Interestingly, at this time point, as shown in **Figure 3.44.**, the vast majority of DCs were CD86⁺ in all groups examined, in contrast to the findings observed at day 10 ($89.3\% \pm 0.76$ vs $15.1\% \pm 2.3$). Moreover, no significant differences were observed between saline and bleomycin challenged mice ($87.7\% \pm 1.1$ vs $91\% \pm 2.2$). In addition, at this later time point, neutralisation of TSLP activity *in vivo* had no effect on CD86 expression by lung DCs in either saline- or bleomycin-challenged mice. This apparent global activation of lung DCs in all mouse groups was confirmed by examining DC OX40L expression. As can be seen in **Figure 3.45**, the majority of DCs in both saline and bleomycin mouse groups also expressed OX40L ($64.3\% \pm 2.8$ vs $66\% \pm 3.0$). However, in contrast to CD86 expression, the administration of 28F12 resulted in a small but significant reduction in DC OX40L expression in both saline and bleomycin-challenged mice ($69.6\% \pm 2.7$ vs $58.6\% \pm 1.5$; $72.1\% \pm 1.3$ vs $60.1\% \pm 2.8$).

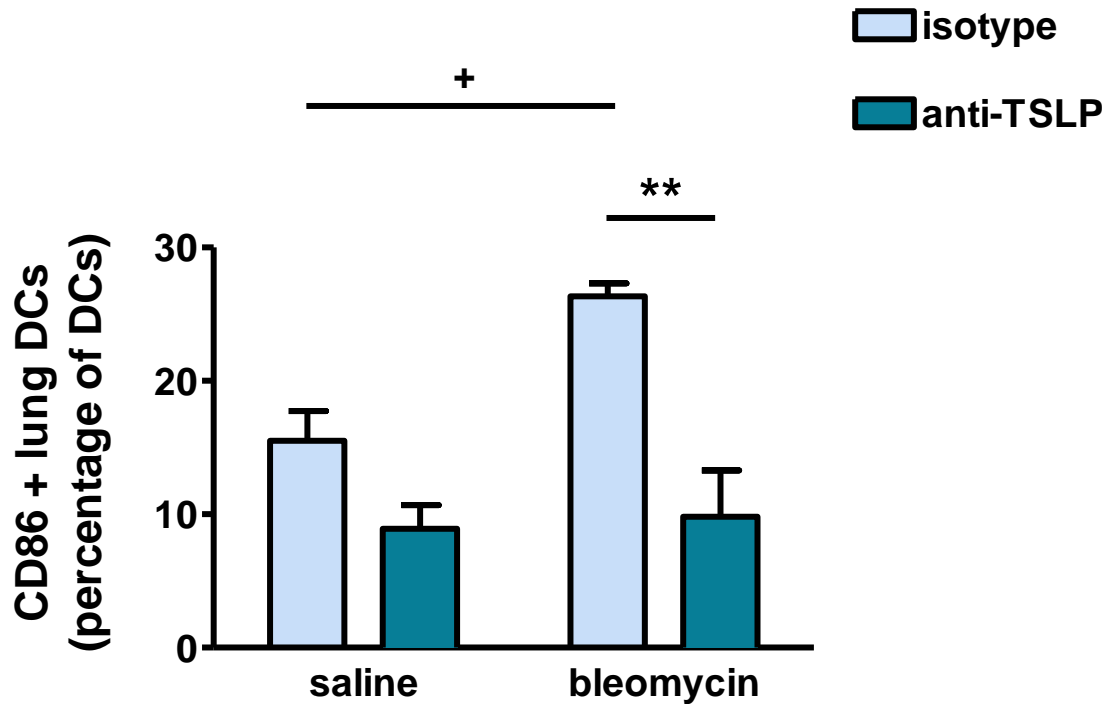


Figure 3.43A.

Administration of anti-TSLP antibody reduces the bleomycin-induced increase in CD86+ lung dendritic cells at day 10.

Figure shows the effect of anti-TSLP administration on CD86 expression by lung DCs following bleomycin-induced lung injury at day 10. C57Bl/6 mice were instilled with saline or bleomycin, and administered isotype control or anti-TSLP antibody i.p. as per **Materials and Methods**. Lungs were harvested for preparation of single cell lung suspensions as described in **Materials and Methods**. Data are presented as percentage of lung dendritic cells (**R1 – Figure 3.41B.**) which are CD86+ \pm SEM; n=3 in all groups; + p<0.05, comparison with saline / isotype; **p<0.01, comparison with bleomycin / isotype; two-way ANOVA.

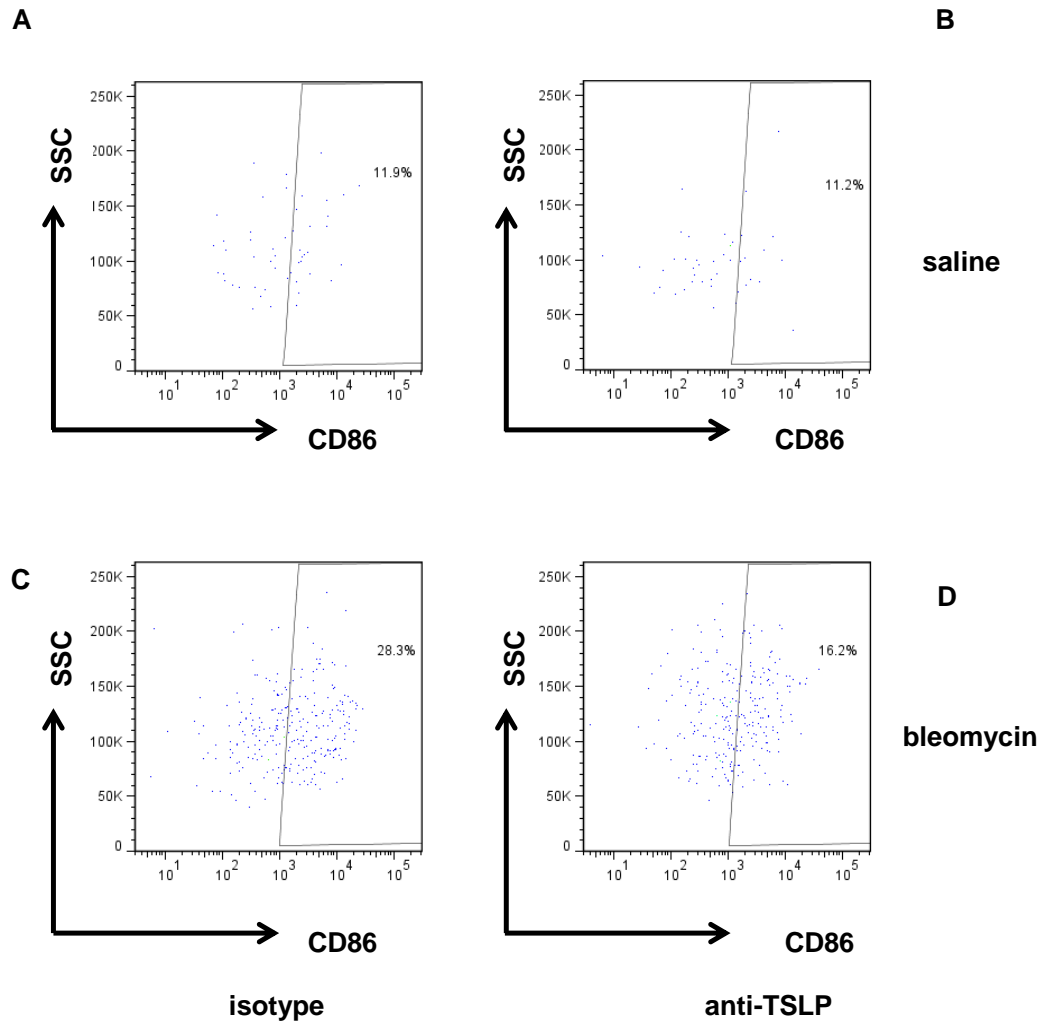


Figure 3.43B.

Administration of anti-TSLP antibody reduces the bleomycin-induced increase in CD86+ lung dendritic cells at day 10.

Figure shows representative FACS plots demonstrating the effect anti-TSLP administration on bleomycin-induced increase in CD86+ DCs in the lung at day 10. C57Bl/6 mice were instilled with saline or bleomycin, and administered isotype control or anti-TSLP antibody i.p. as per **Materials and Methods**. Lungs were harvested for preparation of single cell lung suspensions as described in **Materials and Methods**. CD86 staining was assessed in cells identified as DCs (R1 – Figure 3.41B). (A) saline and isotype control antibody ; (B) saline and anti-TSLP antibody; (C) bleomycin and isotype control antibody; (D) bleomycin and anti-TSLP-antibody; n=3, all groups.

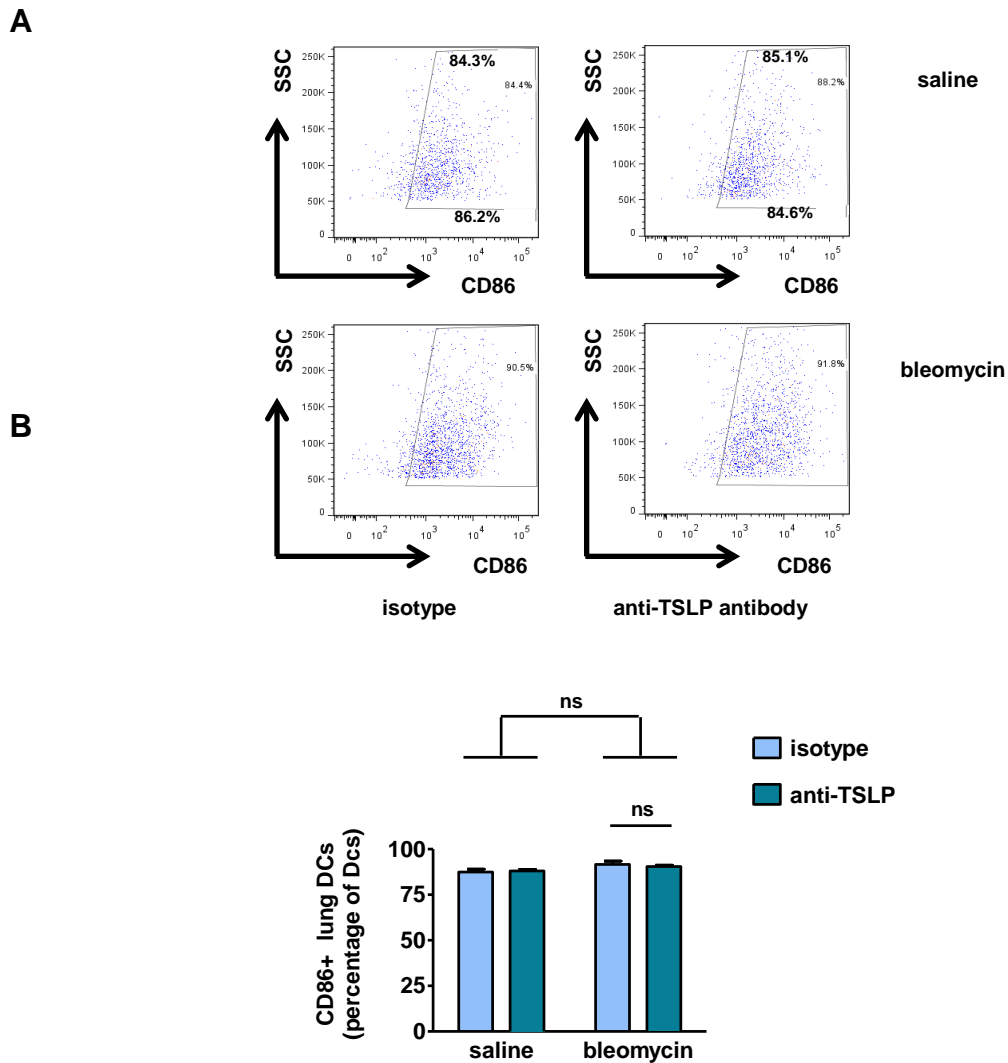


Figure 3.44.

CD86 expression by lung DCs is similar for saline-treated and bleomycin-challenged mice at day 28.

Figure shows the effect of anti-TSLP administration on CD86 expression by lung DCs following bleomycin-induced lung injury at day 28. C57Bl/6 mice were instilled with saline or bleomycin, and administered isotype control or anti-TSLP antibody i.p. as per **Materials and Methods**. Lungs were harvested for preparation of single cell lung suspensions as described in **Materials and Methods**. **Panel A**: representative FACS dot plots obtained for each experimental condition. CD86 staining was assessed in cells identified as DCs (**R1 – Figure 3.42B**). **Panel B**: percentage of DCs which are CD86+; data are presented as percentage of lung DCs (**R1 – Figure 3.42B**) which are CD86+ \pm SEM; $n=3$ in all groups; ns, non-significant; two-way ANOVA.

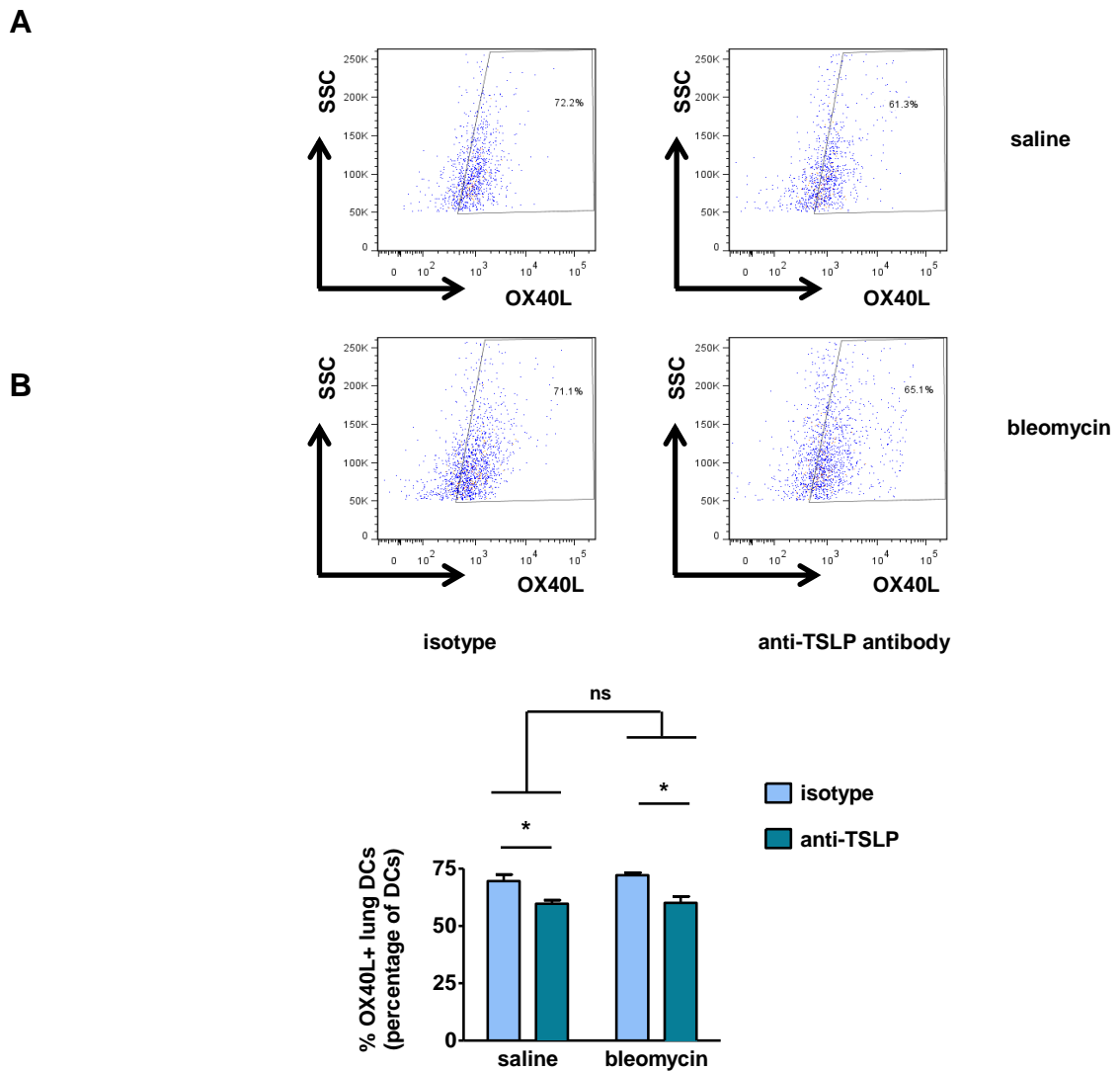


Figure 3.45.

Administration of 28F12 attenuates OX40L expression by lung DCs at day 28 following saline and bleomycin instillation.

Figure shows the effect of anti-TSLP administration on OX40L expression by lung DCs following bleomycin-induced lung injury at day 28. C57Bl/6 mice were instilled with saline or bleomycin, and administered isotype control or anti-TSLP antibody i.p. as per **Materials and Methods**. Lungs were harvested for preparation of single cell lung suspensions as described in **Materials and Methods**. **Panel A**: representative FACS dot plots obtained for each experimental condition. OX40L staining was assessed in cells identified as DCs (**R1 – Figure 3.42B**). **Panel B**: percentage of DCs which are OX40L+; data are presented as percentage of lung DCs (**R1 – Figure 3.42B**) which are OX40L+ \pm SEM; n=3 in all groups; ns, non-significant; *p<0.05, comparison with isotype; two-way ANOVA.

Collectively, these data suggest that the activation of DCs associated with bleomycin-induced lung injury at day 10 is TSLP-dependent. However, the current findings do not allow me to draw any firm conclusions regarding the role TSLP in promoting DC activation during the fibrotic phase of this model. In contrast, they suggest that during the later phase of these extended time-course studies, DC activation may have been influenced by the administration of rat IgG itself, independent of its target specificity. The implications of these findings will be discussed in **Section 4.6.1**.

3.4.5. The effect of bleomycin-induced lung injury on the lung T cell profile at day 7

TSLP-activated dendritic cells (TSLP-DCs) promote the development of T-2 immune responses by inducing the differentiation of Th2 lymphocytes from naïve CD4⁺ cells (Ito *et al.*, 2005), as well as by maintaining a population of effector Th2 cells (Wang *et al.*, 2006). Although the importance of a T-2 immune phenotype in experimentally-induced fibrosis has been previously highlighted (Belperio *et al.*, 2002), the precise profile of Th2 cells following bleomycin-induced lung injury in mice is less clear. Having demonstrated that bleomycin challenge is associated with TSLP-mediated DC activation, at least during the inflammatory phase of this model, I next examined if this response was associated with an increase in the Th2 cell population in the lung. The gating strategy used to identify T lymphocytes is described in more detail in **Materials and Methods**. Briefly, however, lymphocytes were initially gated on forward and side scatter, before CD4, CD8 and TCR- β expression was used to identify sub-populations for further analysis of intracellular cytokine staining.

3.4.5.1. The effect of bleomycin-induced lung injury on T-cell accumulation in the lung

I first examined the T-cell profile in the lung 7 days following bleomycin-induced lung injury. Mice were instilled with bleomycin (2 mg/kg) or saline as described in **Materials and Methods**. Mice were sacrificed at day 7 for FACS analysis of lung single cell suspensions. Data are presented as a percentage of cells identified as lymphocytes on forward and side scatter profile which are TCR- β +ve. As can be seen in **Figure 3.46. (A)**, at this time point, no significant differences were observed in the total $\alpha\beta$ T-cell lung population in bleomycin-challenged mice compared with saline control ($26.2\% \pm 0.7$ vs $20.7\% \pm 3.5$). Moreover, analysis of $\alpha\beta$ T-cell sub-populations (**Figure 3.46. (B-C)**) demonstrates that bleomycin-challenge did not result in any significant change in the CD4⁺ or CD8⁺ populations compared to saline control ($9.8\% \pm 0.4$ vs

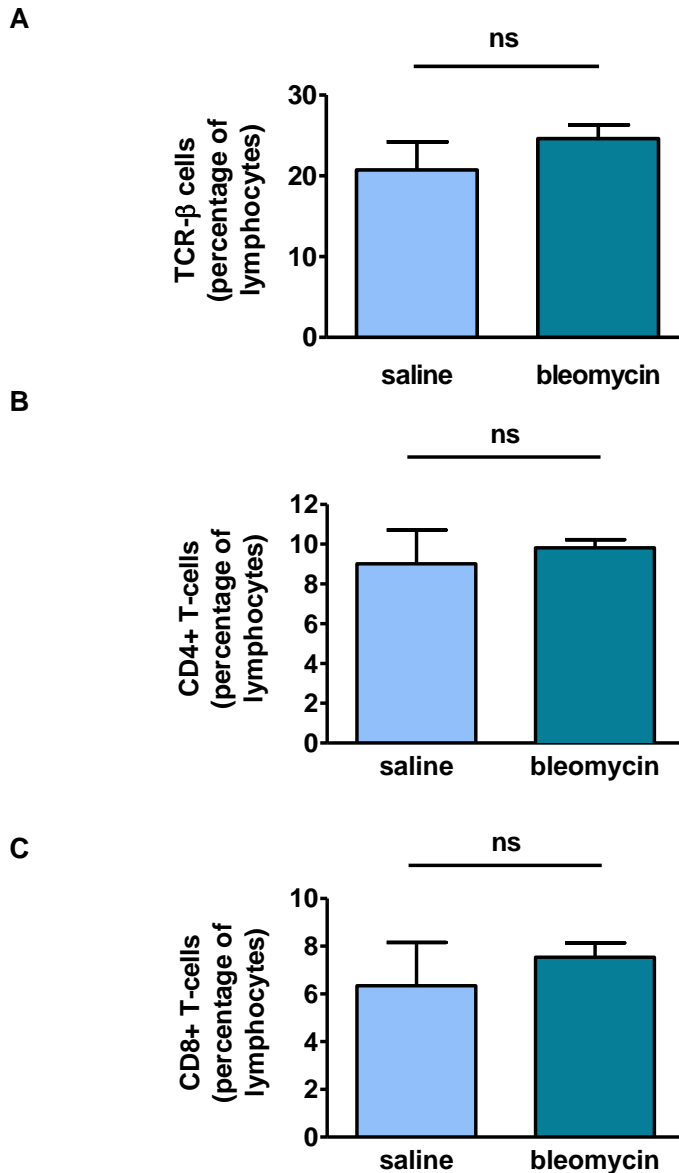


Figure 3.46.

Bleomycin challenge in mice does not result in an increase in the total, CD4 + or CD8 + T-cell lung populations at day 7.

C57Bl/6 mice were instilled with saline or bleomycin, and lungs harvested as previously described. Lungs were harvested at day 7 for preparation of single cell suspensions, as described in **Materials and Methods**, for FACS analysis. **(A)** TCR-β + cells, expressed as percentage of lymphocytes gated on forward and side scatter, in lung homogenates of saline-treated mice or bleomycin-challenged mice; **(B)** CD4+ cells, expressed as percentage of αβ T cells; **(C)** CD8+ expressed as percentage of αβ T-cells; saline, n=3; bleomycin, n=4; data are presented as mean percentage of designated population ± SEM; ns-non-significant, Student's paired t-test.

9.0% \pm 1.7; 7.5% \pm 0.6 vs 6.3% \pm 1.8 respectively; data presented as percentage of cells identified as lymphocytes on forward and side scatter which are TCR- β +, and CD4+ or CD8+). These data suggest that bleomycin challenge does not result in any changes in the total T cell population of the lung, or indeed the proportion of $\alpha\beta$ T-cells which are CD4+ or CD8+. Representative FACS plots for CD4+ and CD8+ cells are presented in the **Appendix A4**.

3.4.5.2. *The effect of bleomycin-induced lung injury on Th1 and Th2 cells in the lung*

Having demonstrated that BLM challenge does not induce any changes in the overall T-cell population, including CD4+ and CD8+ subsets, at day 7 post-injury, I performed further analyses of the CD4+ population to determine if bleomycin-induced lung injury was associated with a polarisation of the T-helper subset towards a T-2 phenotype. Data are presented as percentage of CD4+ / TCR- β + lymphocytes which were either IL4+ or IFN- γ +, markers of Th2 and Th1 cells respectively.

As can be seen in **Figure 3.47.**, bleomycin-challenge resulted in a significant increase in the proportion of CD4+ cells which display a Th2 phenotype (TCR β + / CD4+ / IL-4+) compared to saline control (34.1% \pm 0.8 vs 26.6% \pm 0.7) at day 7 post injury. However, no differences were observed in the Th1 cell population (TCR β + / CD4+ / IFN- γ +) between bleomycin-challenge and saline-treatment (1.3% \pm 0.2 vs 0.9% \pm 0.3) (**Figure 3.48.**). Taken together, these data allowed me to conclude that bleomycin-challenge results in polarisation of the immune response, with respect to T-helper cells, towards a T-2 phenotype at this time point, an effect driven by an increase in the Th2 cell population.

3.4.6. *The role of TSLP in influencing the lung T cell profile following bleomycin-induced lung injury at day 10 and 28*

TSLP has previously been reported to play a critical role in promoting the development of T-2 immune responses in animal models of atopic asthma via its unique activation of DCs. However, its role in promoting such responses in non-allergen driven lung injury is unknown. Having demonstrated that TSLP mediates bleomycin-associated DC activation, I next examined if neutralisation of TSLP activity *in vivo* would prevent the observed polarisation of the T-helper population in the lung. Mice were exposed to the same experimental conditions as described in **Section 3.4.4.** and the intracellular cytokine staining profile of

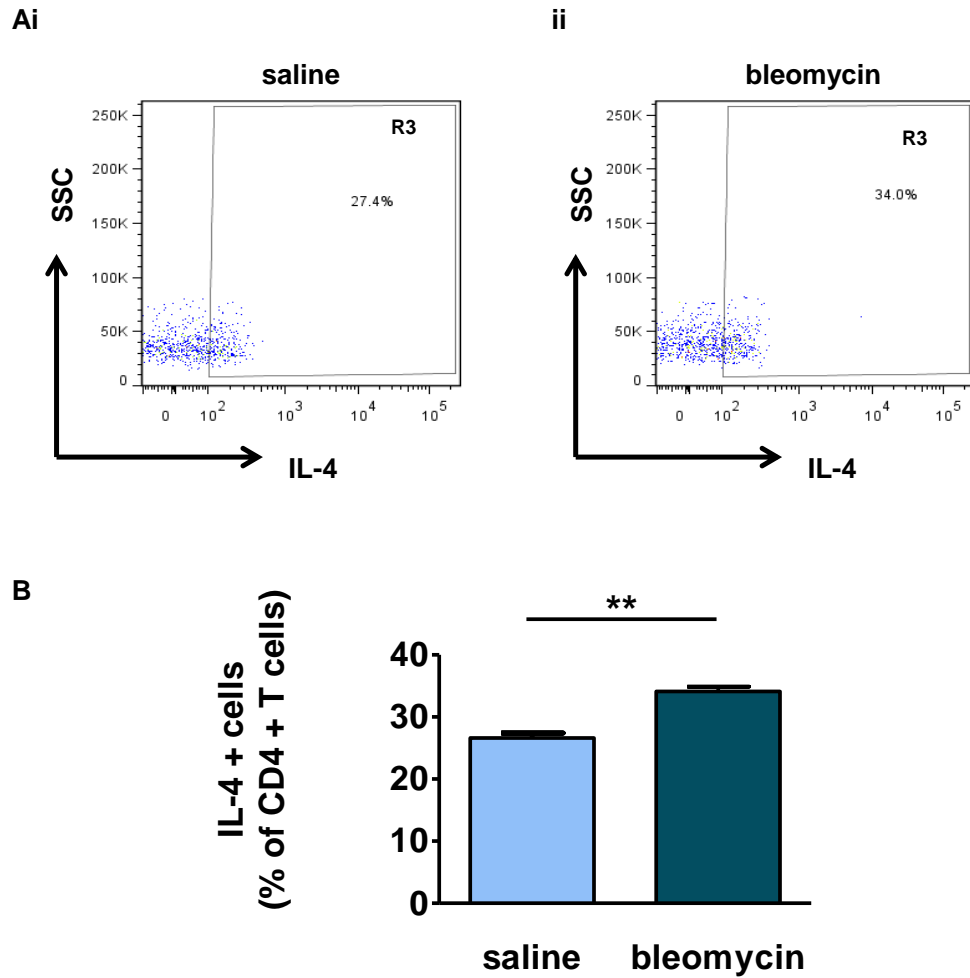


Figure 3.47.

Bleomycin challenge results in an increase in the IL-4 producing CD4 + T cell population in the lung at day 7.

Figure shows the effect of bleomycin-induced lung injury on the Th2 (IL-4+) cell population in the lung. C57/Bl6 mice were instilled with saline or bleomycin and lungs were harvested for preparation of single cell lung suspensions as described in **Materials and Methods**. **(A)** Representative FACS dot-plots obtained from single cell lung suspensions of mice instilled with saline **(i)** or bleomycin **(ii)**. The gating strategy employed to identify TCR- β + / CD4+ cells for analysis of intra-cellular cytokine staining is described in **Material and Methods**. **(B)** Percentage of Th2 cells in single cell lung suspensions following bleomycin challenge **(R3)**. Data presented as the mean percentage of CD4+ $\alpha\beta$ T-cells which are IL-4+ \pm SEM. Saline, n=3; bleomycin, n=4; **p<0.01 Student's paired t-test.

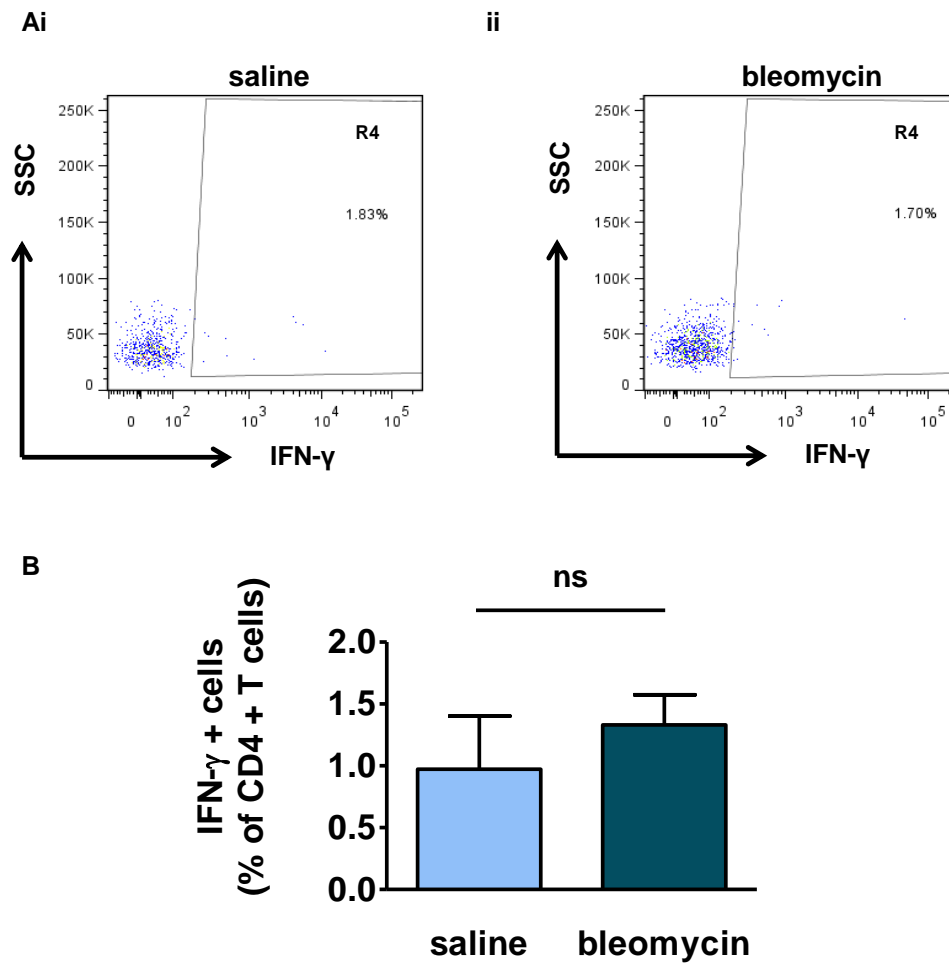


Figure 3.48.

Bleomycin challenge has no effect on the IFN-γ producing CD4 + T cell population in the lung at day 7.

Figure shows the effect of bleomycin-induced lung injury on the Th1 (IFN-γ+) cell population in the lung. C57Bl/6 mice were instilled with saline or bleomycin and lungs were harvested for preparation of single cell lung suspensions as described in **Materials and Methods**. **(A)** Representative FACS dot-plots obtained from single cell lung suspensions of mice instilled with saline **(i)** or bleomycin **(ii)**. The gating strategy employed to identify TCR-β+/CD4+ cells for analysis of intra-cellular cytokine staining is described in **Material and Methods**. **(B)** Percentage of Th1 cells in single cell lung suspensions following bleomycin challenge **(R4)**. Data presented as the mean percentage of CD4+ αβ T-cells which are IFN-γ ± SEM. Saline, n=3; bleomycin, n=4; ns-non significant, Student's paired t-test.

CD4+/TCR- β + cells, as described in **Materials and Methods**, was examined by FACS analysis.

3.4.6.1. *Effect of 28F12 administration on lung CD4+ and CD8+ $\alpha\beta$ T-lymphocytes*

As presented in **Section 3.4.5.1.**, bleomycin challenge did not result in any significant change in the size of CD4+ and CD8+ T-lymphocyte populations, compared with saline control, at 7 days. Further analysis of these cell populations at days 10 and 28 in this model demonstrated that at these later time points, bleomycin challenge was again not associated with any significant differences in the size of these cell populations, compared with saline controls (data presented in **Appendix A5**).

3.4.6.2. *Effect of 28F12 administration on the bleomycin-induced polarisation of the T-helper cell profile towards a T-2 phenotype*

Bleomycin-induced lung injury was demonstrated to be associated with polarisation of the T-helper cell population in the lung towards a T-2 phenotype at day 7 post injury (**Section 3.4.5.2.**). In light of previous reports suggesting persistent polarisation of the immune response during the fibrotic phase of the bleomycin model (Jakubzick *et al.*, 2003), the following series of analyses were performed to determine if the early predominance of Th2 cells in the lung was maintained at later time points. In addition, the role of TSLP in this process was evaluated. Data are presented as the fold increase in percentage of CD4+ T-cells which displayed a Th1 or Th2 phenotype compared to the saline/isotype control group.

As can be seen in **Figure 3.49.**, polarisation of lung CD4+ T-cells towards a T-2 phenotype (IL-4+) was maintained at day 10 (**Panel Ai**: ~7-fold increase in Th2 cells) and during the fibrotic phase of this model at day 28 (**Panel Aii**: ~3-fold increase in Th2 cells) following bleomycin-induced lung injury. In contrast, no significant differences were observed in Th1 cell populations (IFN- γ +) between the saline and bleomycin groups at either day 10 or day 28 (**Panel B**). Representative histograms for these data are presented in **Figures 3.50-3.51.** (days 10 and 28 respectively). The shift of the whole cell population, from lungs harvested at day 28 (**Fig 3.51, panel A**), towards IL-4+ suggests a degree of non-specific staining, and in this regard, one should interpret these data cautiously.

Neutralisation of TSLP activity resulted in an abrogation of this effect at both days 10 and 28 post bleomycin challenge. As demonstrated in **Figure 3.49A**, the fold increase in Th2 cells in bleomycin-challenged mice which received anti-TSLP antibody was not significantly different compared to saline controls at days 10 and 28 (1.03- and 1.28-fold change (**3.49Ai and Aii** respectively)). The administration of anti-TSLP had no effect on the percentage of Th1 cells at either time point (**Figure 3.49Bi-ii**). Collectively, these data therefore suggest that bleomycin-induced lung injury is associated with TSLP-dependent polarisation of T-helper cells towards a T-2 phenotype which is seen as early as day 7, and which is maintained during the fibrotic phase of this model.

No changes were observed in the splenic T-cell profiles of mice challenged with bleomycin compared to saline-treated controls at day 10 (data presented in **Appendix A6**), strongly suggesting that bleomycin-induced lung injury does not result in a systemic immune response, and that the polarisation of CD4⁺ cells observed in this model is a local phenomenon, restricted to the site of initial injury.

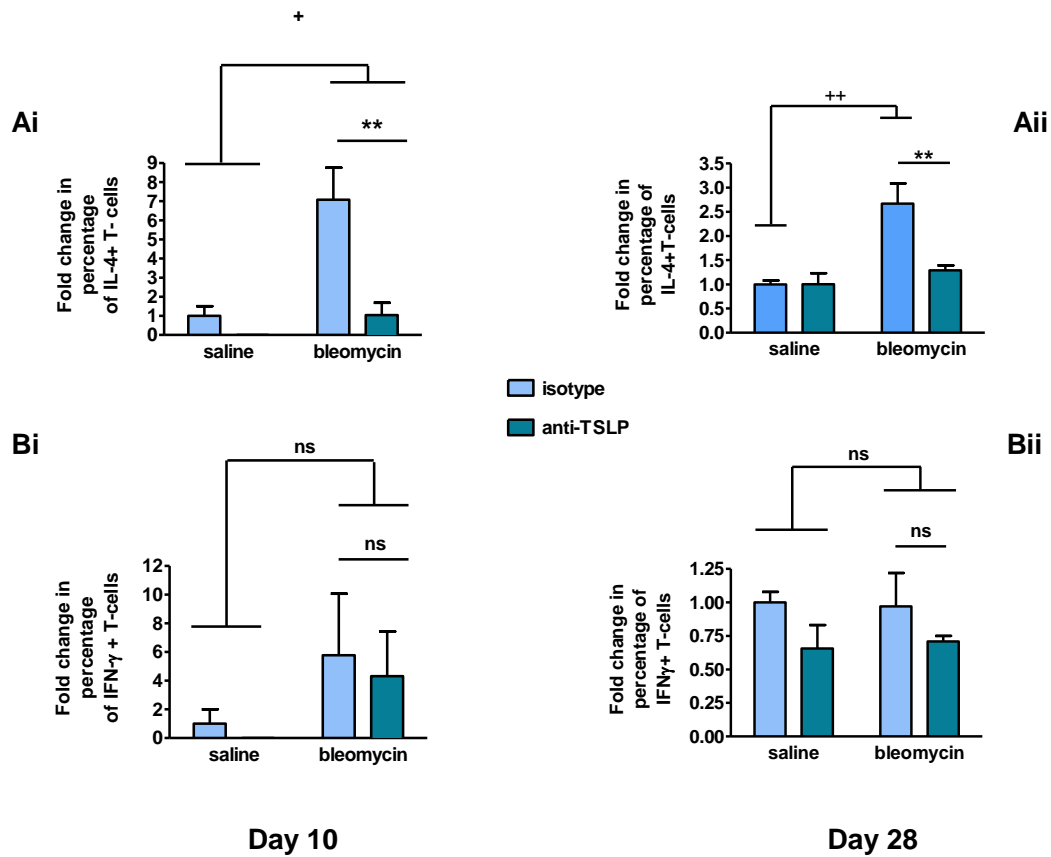


Figure 3.49.

Administration of anti-TSLP antibody reduces the bleomycin-induced polarisation of T-cells in the lung towards a T-2 phenotype at day 10 and 28.

Figure shows the effect of 28F12 administration on Th2 (IL-4+) (**Panel A**) and Th1 cells (IFN- γ) (**Panel B**) following bleomycin-induced lung injury at day 10 and day 28. C57Bl/6 mice were instilled with saline or bleomycin, and administered isotype control or anti-TSLP antibody i.p.; lungs were harvested for preparation of single cell lung suspensions as described in **Materials and Methods**. The gating strategy to identify TCR- β /CD4+ cells for analysis of intra-cellular cytokine staining is described in **Material and Methods**. Data are presented as the mean fold change in percentage of Th2 cells at days 10 and 28 (**Panel Ai-ii**) or of Th1 cells (**Panel Bi-ii**) relative to the saline/isotype group \pm SEM; $n=3$ for all saline groups; $n=5$ and 3 for bleomycin groups (day 10 and 28 respectively). + $p<0.05$; ++ $p<0.01$, comparison with saline; ** $p<0.01$, comparison with bleomycin / isotype; two-way ANOVA.

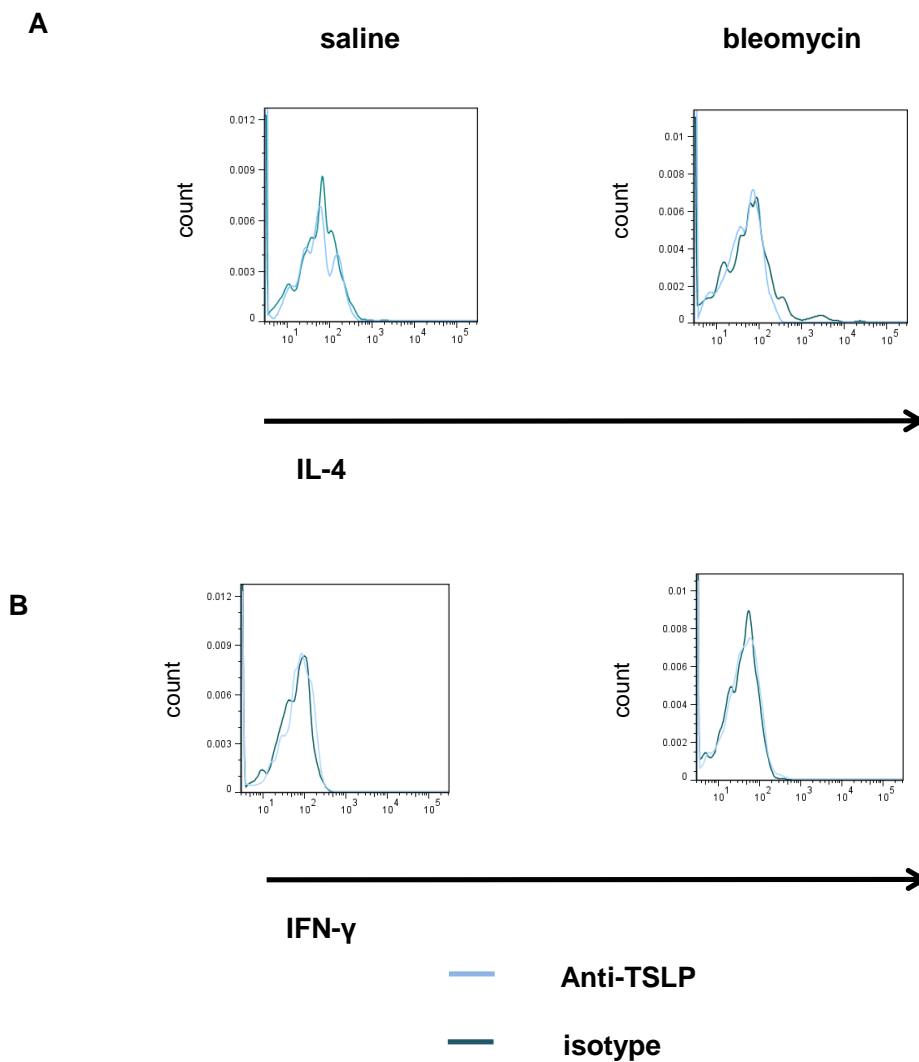


Figure 3.50.

Administration of anti-TSLP antibody reduces the bleomycin-induced polarisation of T-cells in the lung towards a T-2 phenotype at day 10.

Figure shows representative histograms demonstrating the effect of 28F12 administration on the Th2 (IL-4+) and Th1 (IFN- γ +) cell populations in the lung following bleomycin-induced lung injury at day 10. C57Bl/6 mice were instilled with saline or bleomycin, and administered isotype control or anti-TSLP antibody i.p.; lungs were harvested for preparation of single cell lung suspensions as described in **Materials and Methods**. The gating strategy to identify TCR- β + / CD4+ cells for analysis of intra-cellular cytokine staining is described in **Material and Methods**. **(A)** Th2 cells; **(B)** Th1; n=3 for saline, 5 for bleomycin groups.

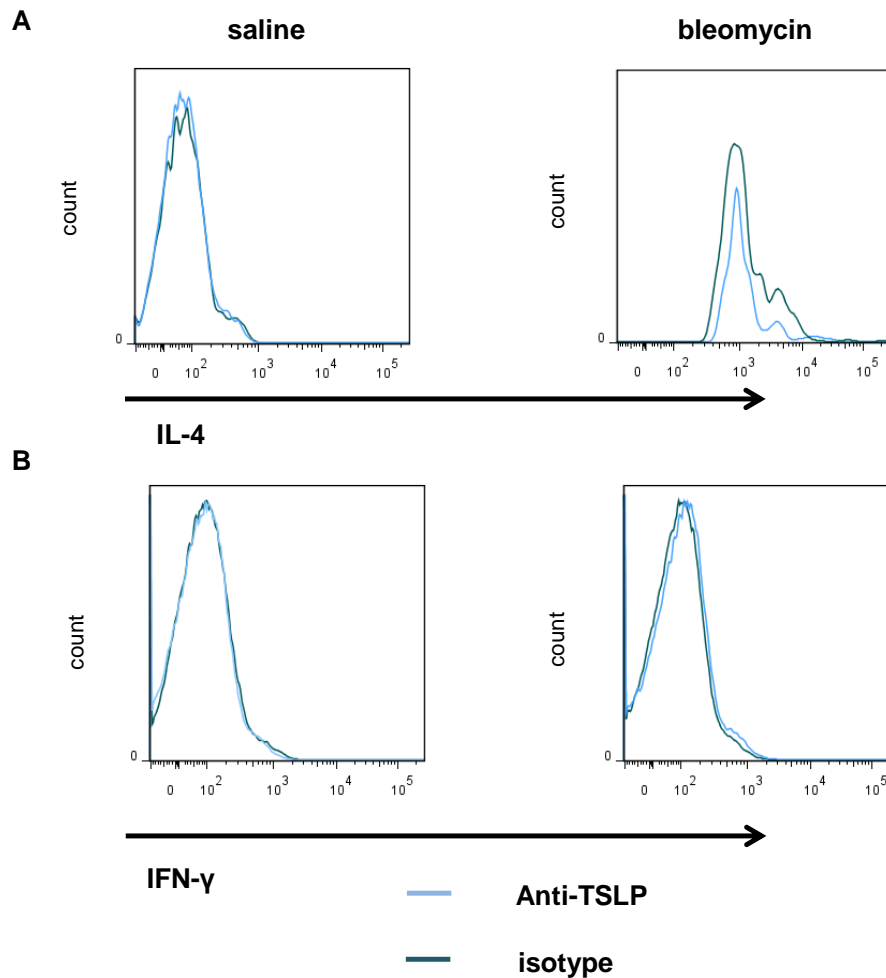


Figure 3.51.

Administration of anti-TSLP antibody reduces the bleomycin-induced polarisation of T-cells in the lung towards a T-2 phenotype at day 28.

Figure shows representative histograms demonstrating the effect of 28F12 administration on the Th2 (IL-4+) and Th1 (IFN- γ +) cell populations in the lung following bleomycin-induced lung injury at day 28. C57Bl/6 mice were instilled with saline with bleomycin, and administered isotype control or anti-TSLP antibody i.p.; lungs were harvested for preparation of single cell lung suspensions as described in **Materials and Methods**. The gating strategy to identify TCR- β + / CD4+ cells for analysis of intra-cellular cytokine staining is described in **Material and Methods**. **(A)** Th2 cells; **(B)** Th1; n=3 for all groups.

3.4.7. *The role of TSLP in the development of bleomycin-induced lung fibrosis*

Previous studies have highlighted the importance of a T-2 immune response following lung injury in amplifying the dysregulated epithelial-mesenchymal crosstalk which contributes to lung fibrogenesis (**reviewed in Section 1.7.1.**). The data presented in the preceding section has highlighted the role of TSLP in promoting DC activation and polarisation of T-helper cells towards a T-2 phenotype following bleomycin-induced lung injury. Having demonstrated significantly greater TSLP immunoreactivity in both human and murine fibrotic lung, compared to control, I sought to determine whether the development of these TSLP-dependent T-2 immune responses were associated with the subsequent development of lung fibrosis. The following series of experiments, therefore, were designed to investigate the pro-fibrotic potential of TSLP in the bleomycin model.

3.4.7.1. *The effect of 28F12 on bleomycin-induced lung collagen deposition*

Extensive studies in the host laboratory employing the bleomycin model to investigate lung fibrosis have demonstrated that significant lung collagen accumulation is observed at 2 weeks following lung injury (Howell *et al.*, 2005; Scotton *et al.*, 2009). Therefore, the effect of neutralising TSLP activity on lung collagen expression at both the mRNA and protein level was examined initially at day 14. Mice were exposed to the same experimental conditions as described in **Section 3.4.1.**, and lungs were harvested for measurement of *Col1A1* mRNA levels and total lung collagen, as described in **Materials and Methods**. To ensure that mice challenged with bleomycin had received appropriate lung injury, body weights were recorded regularly. As can be seen from **Figure 3.52.**, all animals challenged

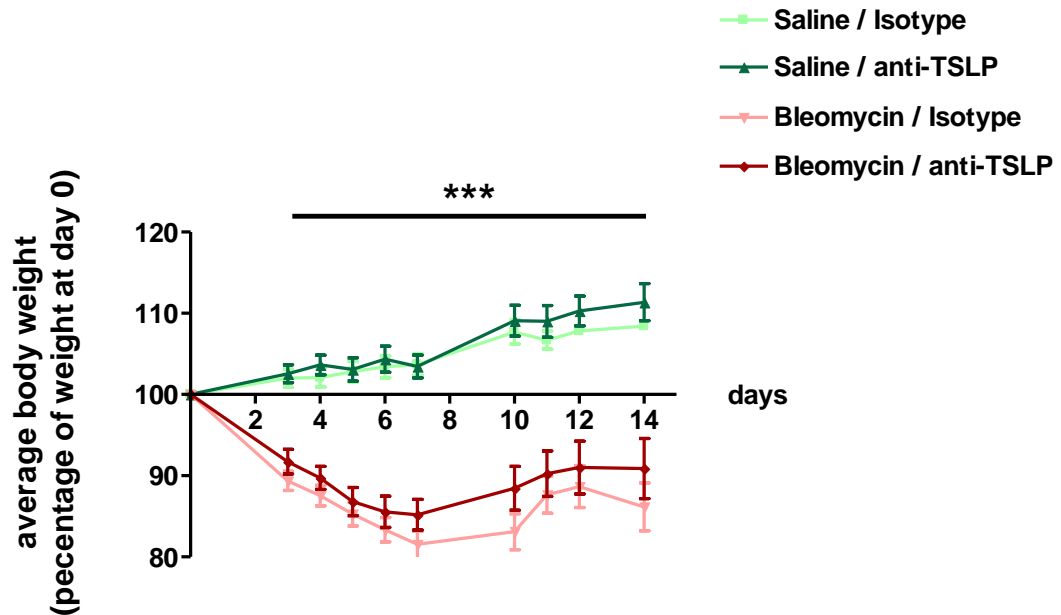


Figure 3.52.

Effect of 28F12 administration on mouse body weights following bleomycin-induced lung injury over 14 days.

Figure shows the effect of 28F12 administration on mouse body weight following bleomycin challenge over 14 days. C57Bl/6 mice were instilled with saline or bleomycin (o.p.), and administered isotype control or anti-TSLP antibody i.p. as per **Materials and Methods**. Results are expressed as mean change in body weight as a percentage of the starting weight. *** $p < 0.001$, comparison between saline and bleomycin; repeated measure ANOVA.

with bleomycin showed a characteristic and significant decrease in body weight from day 3 onwards. Following a nadir at around day 7, body weights in the bleomycin group demonstrated a characteristic recovery. Control mice administered saline displayed weight gain throughout the course of the experiment. Neutralisation of TSLP activity did not attenuate the extent of weight loss in bleomycin-challenged mice (**Figure 3.52.**) Consistent with previous studies (Howell *et al.*, 2005; Krupiczkoj *et al.*, 2008) bleomycin challenge in resulted in a significant increase (~70%) in total lung collagen, as measured by reverse-phase HPLC quantification of hydroxyproline in lung tissue, compared with saline control at day 14 post-injury (**Figure 3.53**). However, this increase was not inhibited by the administration of an anti-TSLP antibody, confirming that, in this model of lung injury, TSLP did not contribute to the development of lung fibrosis at day 14. In conjunction with the data presented in **Section 3.4.6.**, these data also suggest that bleomycin-induced lung collagen deposition at this time point might be independent of the polarisation of T-helper cells in the lung towards a T-2 phenotype.

Lung collagen mRNA levels may represent a more sensitive endpoint in this model, and I therefore looked at changes in *Col1A1* mRNA levels between saline- and bleomycin-instilled mice. **Figure 3.54.**, demonstrates that lung *Col1A1* mRNA levels were significantly elevated in mice challenged with bleomycin (~3-fold increase relative to saline controls). However, the administration of 28F12 had no effect on bleomycin-induced *Col1A1* mRNA levels, indicating that neutralisation of TSLP activity did not affect lung collagen I gene expression at day 14 post injury in this model.

Although differences in lung *Col1A1* mRNA levels from the two bleomycin-challenged mouse groups (anti-TSLP and isotype) were not significant, there was nonetheless a “trend “ to reduction in mice treated with anti-TSLP antibody. Moreover, previous published studies have demonstrated that targeting IL-13 responsive cells from day 21 onwards attenuates lung fibrosis in this model (Jakubzick *et al.*, 2003). In light of the present findings that both TSLP immunoreactivity and the TSLP-mediated Th2 predominance of lung T-cells is maintained at day 28, further studies were performed to determine if neutralisation of TSLP activity and abrogation of the T-2 polarisation would attenuate lung collagen deposition beyond day 14.

In the first instance, an extended time-course experiment was performed to determine if lung collagen accumulation continued beyond 14 days in the bleomycin model employed by the host lab. Mice were challenged with bleomycin or saline and

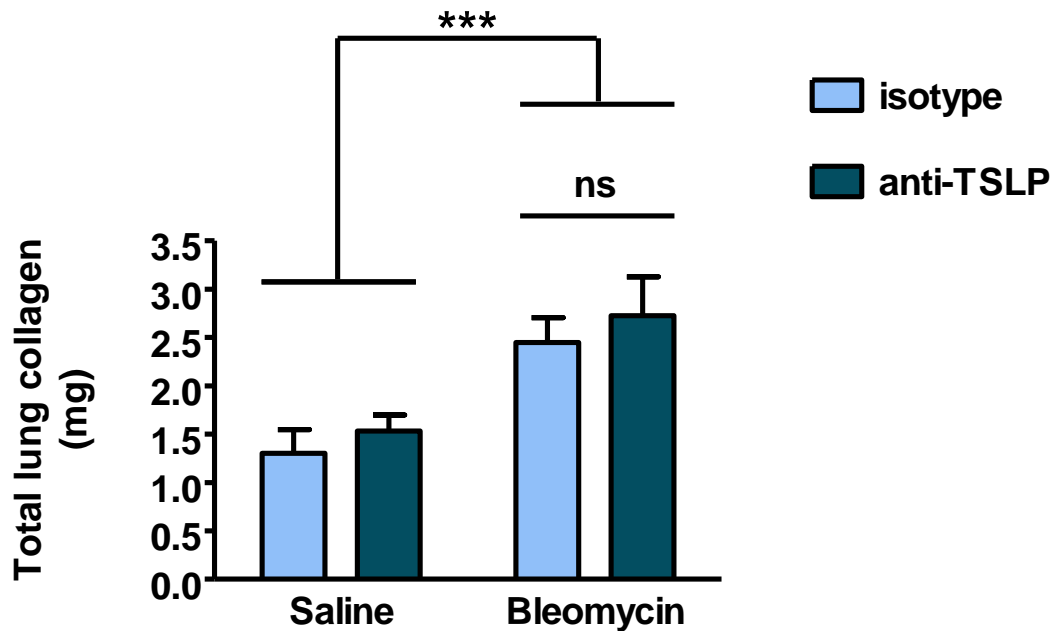


Figure 3.53.

Effect of 28F12 administration on lung collagen accumulation in bleomycin-induced lung injury and fibrosis at day 14.

Figure shows the effect of anti-TSLP administration on bleomycin-induced lung collagen deposition. C57Bl/6 mice were instilled with saline or bleomycin, and administered isotype control or anti-TSLP antibody i.p. as per **Materials and Methods**. Mice were sacrificed at day 14 and lung collagen was measured by reverse phase HPLC quantification of hydroxyproline in acid hydrolysates of pulverised lung. The total amount of collagen in each lung was calculated assuming that lung collagen contains 12.2% w/w hydroxyproline as described in **Materials and Methods**. Data represent the mean \pm SEM, $n=8$, apart from the bleomycin/isotype group, where $n=6$. ns-non significant; *** $p<0.001$; two-way ANOVA.

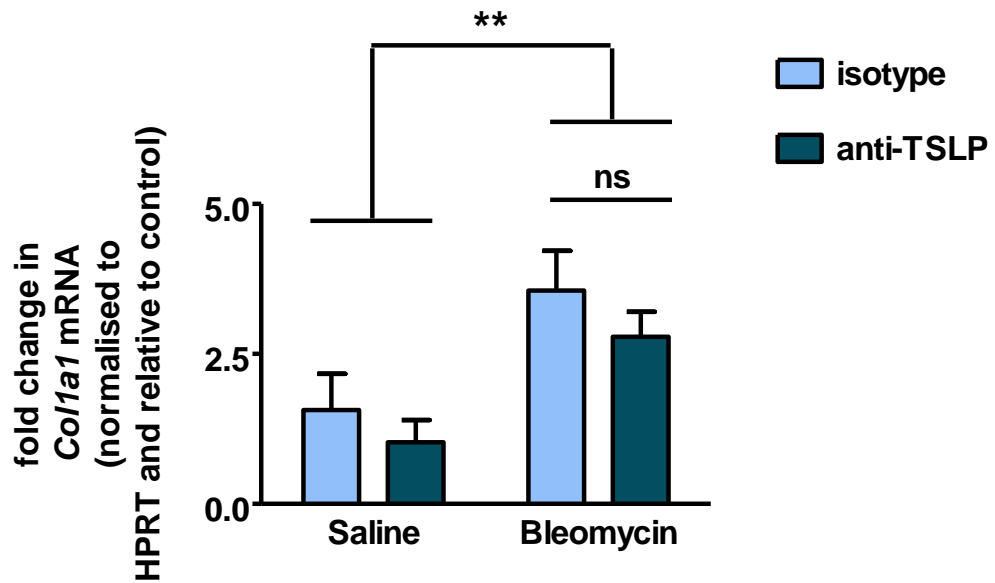


Figure 3.54.

Effect of 28F12 administration on lung *Col1a1* mRNA levels in bleomycin-induced lung injury and fibrosis at day 14.

Figure shows the effect of 28F12 administration on lung *Col1A1* mRNA levels. C57Bl/6 mice were instilled with saline or bleomycin (o.p.), and administered isotype control or anti-TSLP antibody i.p. as per **Materials and Methods**. *Col1a1* mRNA levels were assessed by qRT-PCR of whole lung homogenates. Data are expressed as mean fold-change \pm SEM relative to saline-treated control, normalized to the housekeeping gene *Hprt*. $n=8$ in all groups apart from the bleomycin/isotype group, where $n=6$. ns, non-significant $**p<0.01$, comparison with saline; two-way ANOVA.

total lung collagen was measured at days 7, 10, 14, 21 and 28. As can be seen from **Figure 3.55.**, bleomycin-induced deposition of collagen in the lung continued to increase significantly up to day 28, compared with saline control, with the sharpest increase occurring between days 10 and 21. Mice treated with saline also demonstrate a significant increase in lung collagen at day 28 compared with day 7, which is likely to reflect an aging effect.

Having demonstrated that lung collagen continues to accumulate beyond day 14 and that a T-2 immune response persists at day 28 in this model, I sought to determine if the abrogation of the TSLP-induced Th2 cell predominance in the lung was associated with an attenuation in lung fibrosis at this later time point. For this analysis, mice were randomly chosen from the *in vivo* studies evaluating lung DC and T-cell populations at day 28, as described in **Section 3.4.4. and 3.4.6.** Body weight data from this experiment is presented in the **Appendix (A7)**. As shown in **Figure 3.56.**, total lung collagen was increased by ~80% following bleomycin challenge relative to saline control animals. However, administration of 28F12 had no significant effect on this increase. In conjunction with the findings described in **Section 3.4.6.**, these data therefore suggest that the development of lung fibrosis following bleomycin-induced lung injury is independent of the TSLP-mediated polarisation of T-cells towards a Th2 phenotype.

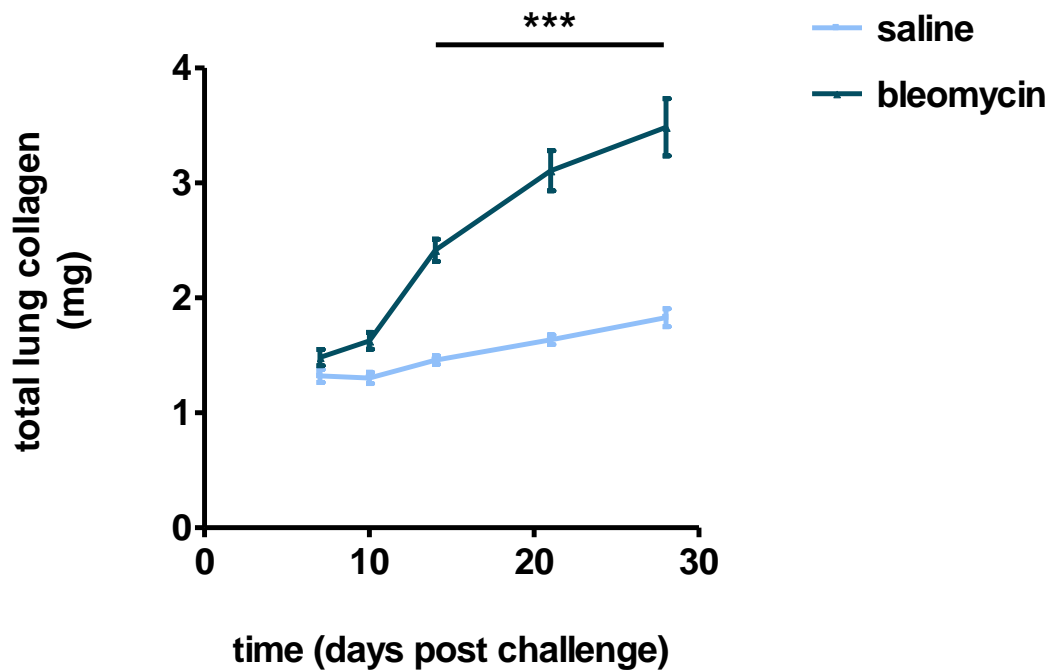


Figure 3.55.

Bleomycin-induced lung injury results in a time-dependent increase in lung collagen accumulation over 28 days.

Figure shows the effect on lung collagen deposition following bleomycin challenge. C57Bl/6 mice were instilled with saline or bleomycin (o.p.); lungs were harvested from mice at varying time-points (days 7, 10, 14, 21 and 28) and lung collagen was measured by reverse phase HPLC quantification of hydroxyproline in acid hydrolysates of pulverised lung. The total amount of collagen in each lung was calculated assuming that lung collagen contains 12.2% w/w hydroxyproline as described in *Materials and Methods*. Data represent the mean \pm SEM; n=10, saline; n=11, bleomycin; ***p<0.001, comparison with saline controls, two-way ANOVA.

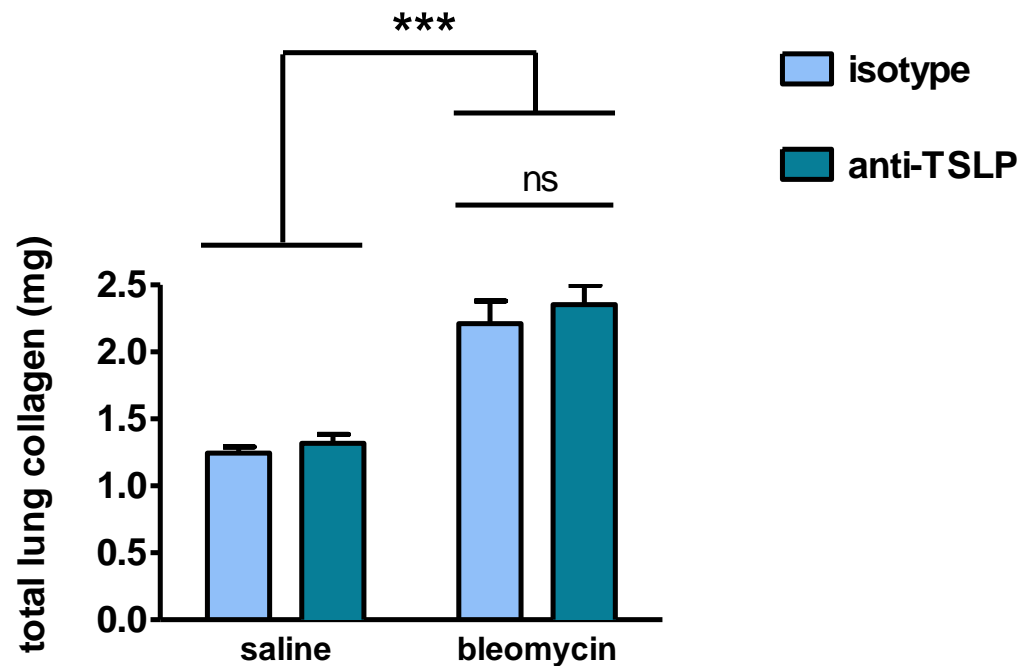


Figure 3.56.

Effect of 28F12 administration on lung collagen accumulation in bleomycin-induced lung injury and fibrosis at day 28.

Figure shows the effect of anti-TSLP administration on bleomycin-induced lung collagen deposition. C57Bl/6 mice were instilled with saline or bleomycin, and administered isotype control or anti-TSLP antibody i.p. as per **Materials and Methods**. Mice were sacrificed at day 28 and lung collagen was measured by reverse phase HPLC quantification of hydroxyproline in acid hydrolysates of pulverised lung. The total amount of collagen in each lung was calculated assuming that lung collagen contains 12.2% w/w hydroxyproline as described in **Materials and Methods**. Data represent the mean \pm SEM, $n=5$, all saline groups; $n=7$, bleomycin/isotype; $n=10$, bleomycin/anti-TSLP; ns-non significant, comparison with isotype; *** $p<0.001$, comparison saline; two-way ANOVA.

3.4.8. Summary

The results described in this chapter, examining the role of TSLP in bleomycin-induced changes in lung immune cell profiles and the development of lung fibrosis, showed that:

- TSLP immunoreactivity, apparent from day 7 and maintained at day 28, is greater in bleomycin-challenged murine lung, compared to saline-treated controls, localising to alveolar epithelial cells (AECs), fibroblasts and macrophages
- TSLPR immunolocalises to AECs, bronchial epithelial cells (BECs), fibroblasts and dendritic cells in murine fibrotic lung;
- bleomycin challenge induced a significant time-dependent increase in the lung dendritic cell population which is independent of TSLP activity;
- bleomycin-induced lung injury was associated with activation of lung dendritic cells at day 10 which was inhibited by the administration of anti-TSLP antibody;
- prolonged antibody dosing in extended time-course studies using this model was associated with general activation of lung DCs;
- bleomycin-induced lung injury was not associated with an increase in the total, CD4+ or CD8+ T-cell populations in the lung at days 7, 10 and 28 but was associated with persistent polarisation of the CD4+ cell population towards a Th2 phenotype;
- neutralisation of TSLP activity significantly inhibited the bleomycin-induced polarisation of the T-helper cell profile towards a T-2 phenotype during both the inflammatory and fibrotic phases of lung injury;
- the administration of anti-TSLP antibody did not attenuate the bleomycin-induced increase in total lung collagen deposition in the lung at day 14 post injury;
- TSLP does not play a role in the development of lung fibrosis following bleomycin-induced lung injury at day 28;

In conclusion, the reported findings suggest that bleomycin-induced lung injury is associated with increased TSLP expression. These data demonstrate for the first time that this injury is associated with predominance of Th2 lymphocytes in the lung, as well as the accumulation of activated DCs. Although DC accumulation appeared

independent of TSLP activity in this model, DC activation and polarisation of the T-helper population was demonstrated to be TSLP-dependent, highlighting a novel role for this cytokine in the development of T-2 immune responses following non-allergen driven lung injury. However, the development of lung fibrosis in this model appears independent of the bleomycin-induced polarisation of T-cells towards a T-2 phenotype. Moreover, the findings described above suggest that TSLP does not contribute to the development of lung fibrosis following bleomycin challenge.

CHAPTER 4: DISCUSSION

Overview

Over the years, numerous animal and human studies have highlighted the importance of a polarised T-2 immune response to injury in amplifying the dysregulated cross-talk between the epithelium and mesenchyme in lung fibrosis (Belperio *et al.*, 2002; Wallace *et al.*, 1995). Redressing this polarisation may offer a more selective immunosuppressive effect than conventional anti-inflammatory therapy and has been demonstrated to attenuate experimentally-induced fibrosis (Jakubzick *et al.*, 2003). This is reflected by a growing interest in the development of such therapeutic strategies in human fibrotic disease, including idiopathic pulmonary fibrosis (IPF). Sustained epithelial injury is regarded as a critical initiating factor in the pathogenesis of IPF (Selman *et al.*, 2002), and it is increasingly recognised that danger signals released from injured epithelial cells are capable of engaging the adaptive immune response following injury (Pulendran, 2004), thus potentially promoting fibrogenesis.

Thymic stromal lymphopoietin (TSLP) has recently emerged as a key cytokine involved in the development of T-2 immune responses *in vivo* (Taylor *et al.*, 2009; Zhou *et al.*, 2005) though this is not a universal finding, as the differentiation of Th2 cells in TSLPR^{-/-} mice infected with the helminth pathogens, *Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis*, is unimpaired (Massacand *et al.*, 2009). Nonetheless, its importance in the pathogenesis of atopic conditions, particularly in the lung, is well recognised (Al-Shami *et al.*, 2005). However, its role in the development of non-allergen driven lung diseases, also characterised by increased expression of T-2 cytokines, such as IPF, is unknown.

Although traditionally regarded as an epithelial-derived cytokine (Soumelis *et al.*, 2002), TSLP expression at the protein level has been reported for a number of other cell types, including airway smooth muscle cells (Redhu *et al.*, 2011). Previous mRNA expression studies suggested that lung fibroblasts may also have the potential to produce TSLP (Soumelis *et al.*, 2002). Lung fibroblasts represent the key effector cell responsible for the excessive deposition of extracellular matrix proteins which leads to the parenchymal fibrosis characteristic of interstitial lung disease (Scotton *et al.*, 2007). However, their close proximity to the epithelium

means that they are also in a unique position to relay signals of epithelial injury to trafficking immune cells, potentially orchestrating local immune responses.

This thesis therefore examined the hypothesis that **the induction of TSLP following lung injury contributes to the development of pulmonary fibrosis by promoting a T-2 immune response.**

To address this hypothesis, initial experiments aimed first to establish whether TSLP is expressed in human disease by examining the cellular localisation of TSLP, and its receptor, in IPF lung by immunohistochemistry. These studies identified the lung fibroblast as a potential cellular source and target of TSLP in this condition. Similar analyses in the bleomycin model of fibrosis confirmed these findings, suggesting for the first time that TSLP expression increases following non-allergen driven lung injury. Subsequent experiments were performed to determine the signalling pathways involved in mediating TSLP expression by primary human lung fibroblasts (pHLFs) *in vitro*. In this regard, a central role for the JNK/c-Jun pathway was highlighted. In addition, the studies performed during the course of this doctoral work demonstrated for the first time that lung fibroblasts express a functional TSLP receptor signalling axis, and that TSLP induces the upregulation of CCL2 via a STAT3-dependent mechanism. TSLP was further shown to be capable of inducing chemotaxis of human monocytes via the generation of this immunomodulatory T-2 chemokine. Thereafter, the role of TSLP in the development of a T-2 immune response and lung fibrosis *in vivo* was examined using a neutralising anti-TSLP antibody in the bleomycin model of lung injury and fibrosis. These studies demonstrated that passive neutralisation of TSLP activity following bleomycin-induced lung injury inhibited the activation of dendritic cells and the concomitant polarisation of the immune response towards a T-2 phenotype. However, this effect was not associated with an attenuation in bleomycin-induced fibrosis, as measured by lung collagen accumulation.

Taken together, these data demonstrate for the first time that:

1. Lung fibroblasts represent both a source and cellular target of TSLP;
2. TSLP contributes to DC activation and the development of T-2 immune responses following bleomycin-induced lung injury;
3. TSLP does not contribute to lung collagen accumulation in this model.

The following sections will discuss these findings and their implications in detail.

4.1. The cellular localisation of TSLP in lung fibrosis

4.1.1. Introduction

Immunohistochemical analysis of tissue sections has proved useful in highlighting potential cellular sources of TSLP T-2 dominated inflammatory conditions in humans. With respect to pulmonary disease, known cellular sources of TSLP include airway epithelial cells and smooth muscle cells (Ying *et al.*, 2008; Zhang *et al.*, 2007). However, these cell types are not felt to contribute significantly to the pathogenesis of lung fibrosis. In murine models of pulmonary disease, TSLP immunoreactivity has only been assessed in a single study localising this cytokine to the bronchial epithelium in a model of airway disease (Nakamura *et al.*, 2008). Despite strong evidence suggesting a predominantly T-2 cytokine profile in IPF (Hancock *et al.*, 1998; Wallace *et al.*, 1995), the potential contribution of TSLP, an important promoter of such responses, has not previously been evaluated in this condition. The findings of the present studies, and their implications, will now be discussed in detail.

4.1.2. Cellular immunolocalisation of TSLP in IPF

The results outlined in **Section 3.1.2.** demonstrate that in non-fibrotic human lung, positive immunostaining for TSLP is restricted to occasional alveolar epithelial cells and macrophages. In contrast, strong TSLP immunoreactivity is consistently observed in lung sections from IPF patients (n=12), localizing to hyperplastic alveolar epithelial cells, as well as spindle-shaped fibroblasts within fibrotic foci, and macrophages. TSLP has previously been localised to the human bronchial epithelium in asthma (Ying *et al.*, 2005). Expression of TSLP has been extensively reported in bronchial and small airway epithelial cells in response to stimuli with biological relevance to exacerbations of human airway disease, including TLR ligands, rhinovirus infection and pollutants (Bleck *et al.*, 2008; Kato *et al.*, 2007). In contrast, in the context of IPF, injury to alveolar, rather than bronchial, epithelial cells is regarded as the primary initiating event for subsequent fibrogenesis, and the demonstration of strong TSLP immunoreactivity for alveolar epithelial cells in lung fibrosis reported in this thesis represents the first such report. Moreover, *in vitro* expression of TSLP by such cells has not previously been reported, apart from in the A549 cell line (Lee *et al.*, 2007a), and will be discussed further in **Section 4.4.**

The identification of strong TSLP immunoreactivity for lung fibroblasts identifies for the first time a cellular source which is common to the pathology of both allergen

and non-allergen driven lung disease. This mesenchymal cell represents the key effector cell responsible for the excessive deposition of extracellular matrix protein leading to interstitial fibrosis (Scotton *et al.*, 2007), as well as the airway remodelling characteristic of chronic asthma (Homer *et al.*, 2005). The current observations are compatible with recent studies identifying fibroblasts as potentially important cellular sources of TSLP in non-pulmonary conditions such as nasal polyposis (Kimura *et al.*, 2011). Moreover, lung fibroblasts have been reported to have the potential to express TSLP *in vitro* (Soumelis *et al.*, 2002), though at the start of this PhD, expression at the protein level had not been reported. The current studies, therefore, suggest that the lung fibroblast may represent an important, and hitherto unrecognised, cellular source of TSLP in several conditions, including pulmonary diseases, characterised by the increased expression of T-2 cytokines.

Strong TSLP immunoreactivity was also observed for mononuclear cells organised within peri-bronchiolar and peri-vascular aggregates in human IPF. These aggregates consist chiefly of T lymphocytes, as well as dendritic cells, suggesting a role for the adaptive immune system in the pathogenesis of IPF (Marchal-Somme *et al.*, 2007a). However, TSLP expression by lymphocytes has not been reported. In contrast, it is well recognised that human lymphocytes express the TSLP receptor (Rochman *et al.*, 2007). Indeed, recent studies have demonstrated that TSLP may induce programmes of proliferation and differentiation in activated T-cells directly (Rochman *et al.*, 2007), in addition to via dendritic cells. The observed immunoreactivity for such cells may therefore reflect localisation of extracellular TSLP bound to its receptor on lymphocytes. The T-cells that accumulate in such follicular structures in IPF are felt to be recently activated, as determined by increased expression of CD45L (Marchal-Somme *et al.*, 2006). More recently, dendritic cells themselves have been reported to upregulate the expression of TSLP in response to TLR ligands *in vitro* (Kashyap *et al.*, 2011). TSLP represents an extremely potent activator of DCs into a T-2 polarising phenotype (**Section 1.4.6.**). These recent findings may therefore reflect an autocrine signalling loop allowing amplification of an immune polarising signal at mucosal sites and / or a second pathway for the development of such responses, circumventing the need for alternative cellular sources of this cytokine. However, despite strong TSLP immunoreactivity for TSLP observed within lymphoid aggregates, due to the time constraints of the current studies, the confident immunolocalisation of TSLP to dendritic cells within these structures was not possible – such studies would represent an important part of future work in this area.

In the present IPF studies, no convincing TSLP immunoreactivity for human airway smooth muscle cells (HASMCs) was observed in contrast to previous reports in chronic obstructive pulmonary disease (COPD) (Zhang *et al.*, 2007). Aside from technical variations, one possible explanation for these differences may be that TSLP immunoreactivity for this cell type was evaluated in biopsies derived from inflamed airways in COPD, which are not characteristic features of IPF.

4.1.3. Cellular immunolocalisation of TSLP following bleomycin-induced lung injury

To determine if a similar pattern of cellular localisation was apparent in experimentally-induced fibrosis, immunohistochemical analysis was undertaken of lungs harvested from mice challenged with bleomycin. The bleomycin model represents the most widely used model for experimentally-induced lung fibrosis, and is described in detail in **Section 1.3.1**.

The results presented in **Section 3.4.2** demonstrate that immunoreactivity for TSLP in the lungs of saline-treated control mice is relatively weak at all time points evaluated from days 3 - 28 and is limited to occasional alveolar epithelial cells and macrophages, a pattern reminiscent of non-fibrotic human lung. In contrast, bleomycin challenge results in a time-dependent increase in TSLP immunoreactivity. At early time points, the pattern of immunoreactivity is similar to saline-treated animals. However, from day 7 onwards, the signal strength increases. In particular, the appearance of fibrotic lesions from day 10 post-challenge enabled the identification of fibroblasts as potential cellular sources of this cytokine, in addition to epithelial cells and macrophages. The observation that TSLP immunoreactivity increases in a time-dependent manner following bleomycin challenge is consistent with a role for TSLP in modulating of the immune response following epithelial cell injury in this model. The detection of TSLP at a time coincident with the onset of dendritic cell and T-lymphocyte recruitment (Bantsimba-Malanda *et al.*, 2010; Izbicki *et al.*, 2002), key cellular targets for TSLP, raised the possibility that this cytokine may play a potential pathogenic role in this model. In contrast to human lung, however, TSLP immunoreactivity for mononuclear cells was not apparent in bleomycin-injured murine lung; this observation is more consistent with current evidence which has not identified the lymphocyte as a cellular source of TSLP *in vivo* or *in vitro*. In all other respects, however, the pattern of TSLP immunoreactivity in murine fibrotic lung is similar to that observed in human IPF

lung, suggesting that this cytokine may contribute to the pathogenesis of lung fibrosis. This concept will be further explored in **Section 4.3**.

4.2. The cellular localisation of TSLP receptor in lung fibrosis

As discussed in **Section 1.4.2.**, the cellular effects of TSLP are initiated following ligand binding to the TSLP receptor complex and activation of downstream STAT signalling pathways. Quantitative PCR on a panel of human cDNA libraries has demonstrated TSLPR expression *in vitro* on a wide variety of cell populations of haematopoietic lineage, including dendritic cells and T lymphocytes (Reche *et al.*, 2001). Evaluation of human TSLPR expression *in vivo* has been hampered by technical difficulties relating to antibody specificity. However, recent studies have demonstrated strong TSLPR immunoreactivity for epithelial cells and smooth muscle cells in airways disease (Semlali *et al.*, 2010; Shan *et al.*, 2010). In contrast, localisation of TSLPR has not previously been examined in fibrotic lung disease, and this will now be discussed in more detail.

4.2.1. Cellular immunolocalisation of TSLPR in IPF and bleomycin-induced fibrosis

The results presented in **Section 3.1.2.** demonstrate strong immunoreactivity TSLPR to bronchial epithelial cells (BEC) and macrophages in normal human lung. In addition, a weaker signal is observed for alveolar epithelial cells (AECs). A similar pattern of immunoreactivity was observed in the lungs of control mice given i.t. saline, though the BEC signal was far less apparent. In contrast, in IPF lung, the range of cells localising TSLPR was more extensive. In addition to AECs, mesenchymal cells, such as smooth muscle cells and fibroblasts, were also highly immunoreactive for TSLPR. These data support the notion that expression of the TSLP receptor is not limited to cells of haematopoietic lineage, and the functional implications of these findings will be discussed in **Section 4.5**. In addition, lymphoid aggregates, which in IPF have previously been demonstrated to consist of lymphocytes and dendritic cells, also demonstrated strong immunoreactivity for TSLPR. Within these structures, the high nuclear to cytoplasmic ratio of lymphocytes did not permit clear cell surface localisation of TSLPR to these cells, in contrast to cells with the morphology of dendritic cells. This pattern of surface localisation for TSLPR was also observed in the lungs of mice challenged with bleomycin, suggesting that the range of cells potentially capable of responding to

TSLP in fibrotic lung is similar in humans and mice. The significance of these findings will now be discussed.

4.2.2. TSLPR immunolocalisation to immune cells in lung fibrosis

The initial identification of TSLP as a factor in thymic stromal media (Friend *et al.*, 1994) influenced much of the early studies evaluating cellular responses to this cytokine, particularly examining the role of this cytokine in lymphocyte development. However, TSLP does not seem critical for B cell development in mice (Carpino *et al.*, 2004) in contrast to T-cell lymphopoiesis and haematopoiesis (Al-Shami *et al.*, 2004). The discovery that immature DCs are potently activated by TSLP into a T-2 promoting phenotype has evoked much speculation that this cytokine may represent a master switch, upstream of IL-4, in the development of pathological inflammatory responses characterised by increased T-2 cytokine expression (Ito *et al.*, 2005)

The demonstration of strong TSLPR immunoreactivity for DCs in human and murine fibrotic lung is consistent with the notion that TSLP-DCs may contribute to the pathogenesis of lung fibrosis by inducing a population of Th2 cells. The relevance of strong TSLPR immunoreactivity for lymphocytes in lung fibrosis is also of particular interest in light of increasing recognition that TSLP may exert direct proliferative and differentiating effects on T-cells themselves (Omori *et al.*, 2007; Rochman *et al.*, 2007). These concepts suggest multiple pathways for TSLP to induce and maintain a population of Th2 effectors cells at sites of injury, and will be discussed in more detail in **Section 4.3.3.**

4.2.3. TSLPR localisation to structural cells in lung fibrosis

It is increasingly recognised that expression of a functional TSLP receptor is not limited to cells of a haematopoietic lineage (Semlali *et al.*, 2010; Shan *et al.*, 2010). The strong TSLPR immunoreactivity displayed by epithelial cells in both IPF and bleomycin-induced fibrosis in the present studies is consistent with recent reports suggesting a similar pattern in lung biopsies from human asthmatic lung (Semlali *et al.*, 2010). Moreover, lung epithelial cells are capable of responding to TSLP in a functional manner *in vitro* - epithelial cell proliferation and repair is enhanced following exposure to TSLP in an IL-13 dependent manner (Semlali *et al.*, 2010). Dysregulated epithelial cell proliferation following lung injury is felt to be central to the pathogenesis of IPF, and it is tempting to speculate that normal wound healing responses become increasingly aberrant in the face of persistent TSLP exposure.

In addition to epithelial cells, the present studies also demonstrated for the first time localisation of TSLPR to mesenchymal cells in IPF, including fibroblasts, suggesting that this cell type, critical to fibrogenesis, may express a functional TSLP-TSLP receptor signalling axis. The potential significance of these findings was explored *in vitro* by examining the expression and function of the TSLP receptor by pHLFs and will be discussed in detail in **Section 4.5**.

4.3. Potential role for TSLP in lung fibrosis

In light of the observations that both human and murine lung fibrosis is associated with increased immunoreactivity for TSLP and TSLPR, the potential role of this T-2 promoting cytokine in fibrogenesis will now be discussed (**Figure 4.1**).

4.3.1. *TNF- α* and TSLP in lung fibrosis

Expression of TSLP has been reported in response to a number of mediators (**Section 1.4.3**). It is of note that consistent upregulation of TSLP expression has been reported across a number of cell types in response to stimulation with TNF- α (**see Table 1.3**). The role of this “early wave” alarm type cytokine in the pathogenesis of IPF was discussed earlier (**Section 1.8.2**). Briefly, in lung fibrosis, injured and reparative epithelial cells are felt to be important sources of this cytokine (Nash *et al.*, 1993; Oikonomou *et al.*, 2006). The mechanisms through which TNF- α exerts its pro-fibrotic effects are multiple and include the upregulation of TGF- β activity (Sullivan *et al.*, 2005). In addition, the capacity of TNF- α to promote lymphocyte recruitment in animal models of lung fibrosis is well recognised (Miyazaki *et al.*, 1995; Oikonomou *et al.*, 2006; Sime *et al.*, 1998). These effects are likely to be mediated by secondary mediators of immune cell recruitment, survival and differentiation including mesenchyme-derived chemokines such as CCL2 (Ohmori *et al.*, 1993). However, the role of TNF- α in an adaptive immune response to tissue stress is not merely limited to cell recruitment. Previous studies have demonstrated an important function for this pleiotropic cytokine in the development of inflammatory responses characterised by increased expression of T-2 cytokines. TNF-receptor knockout mice are unable to mount protective T-2 immune responses and are therefore highly susceptible to *Trichuris* infection (Artis *et al.*, 1999). The mechanisms underlying these effects remain unknown but have been postulated to include downstream effects on immune cells to augment T-2 effector cell responses.

In light of this evidence, it was very interesting to observe the close structural proximity of injured, hyperplastic epithelium and fibroblasts, strongly

immunoreactive for TNF- α and TSLP respectively, to dendritic cells within lymphoid aggregates in serial sections of human IPF lung. Such proximity suggests there could be a functional interaction between these cell types, which may ultimately lead to the development of a pro-fibrotic T-2 microenvironment. One may postulate that, following epithelial injury, increased levels of epithelium-derived TNF- α would promote TSLP expression. Increased local concentrations of TSLP may then serve to polarise the immune response towards a T-2 phenotype. This may occur indirectly, via dendritic cells, or through direct effects on T-cells, and these two potential mechanisms will now be explored in detail with respect to the pathogenesis of IPF.

4.3.2. Potential role of TSLP-activated DCs in lung fibrosis

Recent studies have demonstrated that the IPF lung is heavily infiltrated by immature DCs (**Section 1.5.**). It has been proposed that, following recruitment to the lung from the vasculature along stromally-derived chemokine gradients, these cells are activated locally (Marchal-Somme *et al.*, 2007b), though the mediators responsible for such maturation remain undefined. The interaction between activated DCs and naïve T-cells, resulting in T-cell priming and induction of differentiation, has historically been viewed to occur in local lymph nodes (von Andrian *et al.*, 2000) to which maturing DCs traffic following initiation of maturation. Within this paradigm, it has traditionally been felt that only activated and/or memory T-cells access non-lymphoid peripheral tissue (Mackay *et al.*, 1990) reflecting differential expression of tissue-specific chemokine receptors by such cells (Ebert *et al.*, 2005). In this context, one may hypothesise that TSLP-DCs, activated following epithelial injury, migrate to draining lymph nodes to initiate a programme of T-2 differentiation in naïve CD4⁺ T-cells. Thereafter, these activated Th2 cells, now expressing appropriate chemokine receptors, may traffic to the site of injury along chemokine gradients generated locally, with the subsequent development of a local pro-fibrotic T-2 cytokine milieu. This hypothesis is consistent with previous reports in the literature demonstrating that infiltrating T-cells in IPF are of an activated and memory phenotype (Marchal-Somme *et al.*, 2006). Moreover, mediastinal lymphadenopathy is an increasingly recognised feature of idiopathic interstitial pneumonias, including IPF, though there remains uncertainty as to the pathological importance of these findings (Jung *et al.*, 2000; Souza *et al.*, 2006). It is tempting to speculate that this enlargement reflects, at least in part, a programme of proliferation and differentiation of naïve CD4⁺ T-cells following interaction with TSLP-DCs. However, such a hypothesis remains purely speculative at this time,

and further studies are required to evaluate the cellular composition of locally enlarged lymph nodes in IPF, in particular, focusing on the precise immunophenotype of constituent lymphocytes and DCs.

In addition to immature DCs, IPF lung has been reported to be consistently infiltrated with mature DCs, organised within structures resembling lymphoid follicles (Marchal-Somme *et al.*, 2006). The studies performed during the course of this thesis demonstrate that bleomycin-induced lung injury is associated with an increase in the proportion of activated DCs within the lung. These findings are consistent with a recent study published during the course of this thesis (Bantsimba-Malanda *et al.*, 2010). It is generally accepted that activated DCs do not traffic back to organs from lymph nodes. The present observations therefore suggest that resident or recruited immature DCs mature locally at sites of epithelial injury, following activation. Interestingly, locally released danger signals recently implicated in the pathogenesis of IPF, including uric acid and eATP (Gasse *et al.*, 2009; Riteau *et al.*, 2010), are potent activators of DCs, resulting in the subsequent development of T-2 immune responses. eATP, much like TSLP, induces the maturation of DCs into an IL-12_{lo} phenotype, thus creating a local T-2 permissive microenvironment. Therefore, it is possible that TSLP-DCs interact with activated and memory T-cells locally to perpetuate a T-2 dominant environment. In this regard, long-lived memory Th2 cells have previously been proposed to play key roles at sites of T-2 inflammation (Holt *et al.*, 1999; Mojtabavi *et al.*, 2002). Moreover, TSLP-DCs have been demonstrated to be capable of inducing an expansion of Th2 memory cells, which retain their Th2 commitment and capacity for effector cytokine function (Wang *et al.*, 2006). Thus, TSLP may play a key role in maintaining the T-2 phenotype of recently recruited Th2 effector memory cells at sites of inflammation, thereby favouring the persistence of a microenvironment characterised by increased expression of T-2 cytokines. Such an interaction may be antigen-specific or related to bystander activation of memory T-cells primed during previously unrelated antigenic exposure, as discussed later in this section.

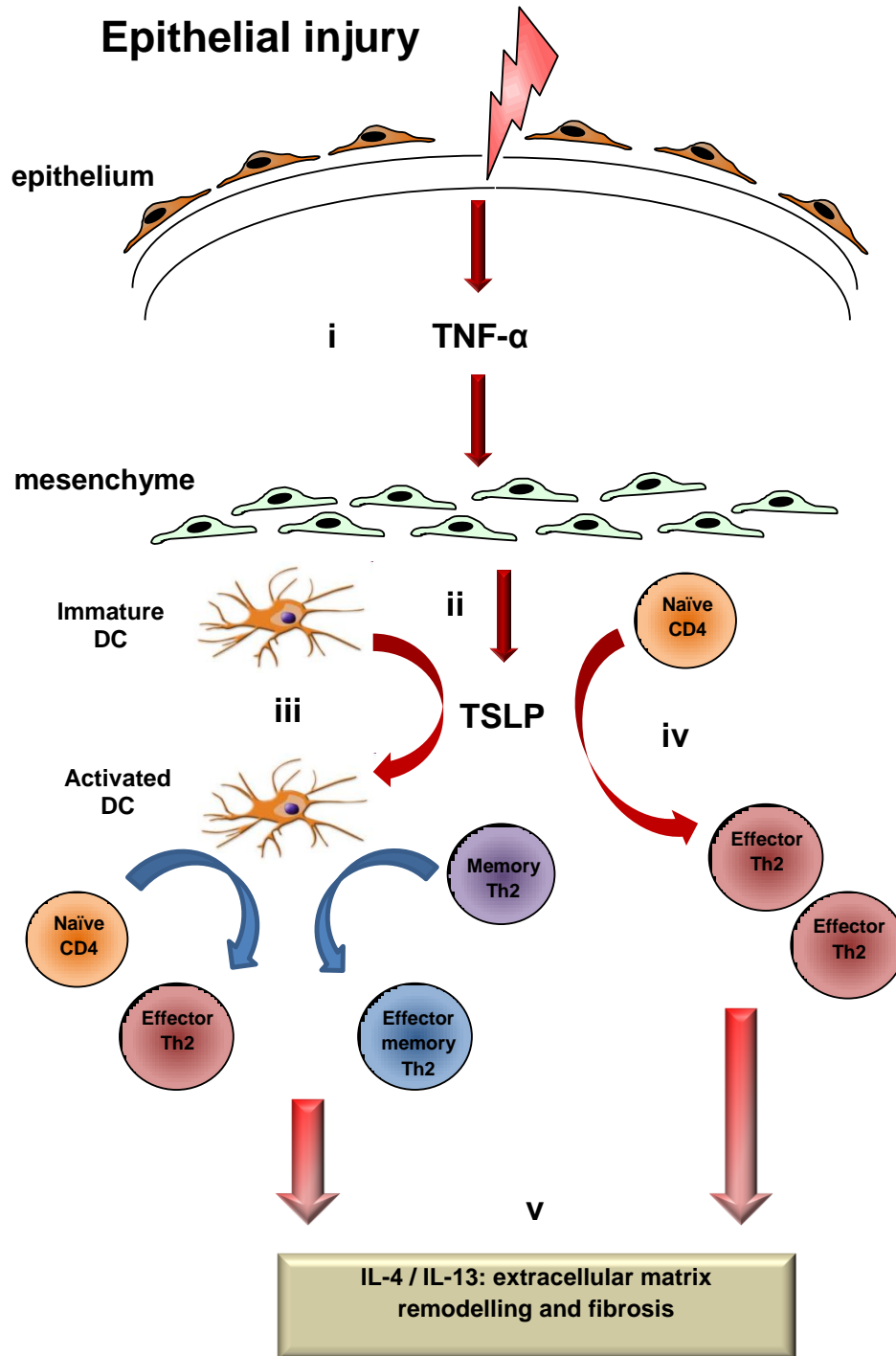


Figure 4.1. Proposed model for fibroblast-derived TSLP in the pathogenesis of lung fibrosis. TNF- α , released from injured epithelium (i), upregulates expression of TSLP by underlying fibroblasts (ii). TSLP promotes the development of a T-2 immune response, indirectly via the activation of dendritic cells (DC) (iii), or via a direct effect on T-cells (iv) in an antigen-specific manner. Engagement of naïve T-cells has traditionally been viewed to occur in the lymph nodes. However, as discussed in text, this standpoint has recently been challenged in light of recent evidence suggesting the potential importance of peripheral naïve CD4⁺ T-cells. A polarised T-2 immune response is characterised by increased expression of pro-fibrotic cytokines such as IL-4 and IL-13 (v).

As alluded to above, naïve CD4⁺ T-cells have not previously been regarded as a significant component of the overall T-cell population in non-lymphoid peripheral tissue, and their re-circulatory pathways have been thought to involve the lymphatic system exclusively. However, this concept has recently been challenged. Naïve T-cells have been reported to be present in non-lymphoid organs in the context of inflammation (Krakowski *et al.*, 2000; Weninger *et al.*, 2003). More recently, significant numbers of naïve T-cells have been shown to traffic through peripheral tissue, including the lung, in a chemokine receptor-independent manner (Cose *et al.*, 2006). Although the fraction of naïve T-cells in non-lymphoid organs is relatively low, it has been suggested that any such cell is likely to traffic through all such organs several times during its lifetime (Westermann *et al.*, 2001). The precise function of naïve T-cells outside of lymph tissue remains unclear, though in light of their rapid transit, it has been proposed that they may play a role in immunosurveillance and the induction of peripheral tolerance [reviewed in (Cose, 2007)].

It is, therefore, also possible that naïve CD4⁺ T-cells may be primed and activated *in situ*, a concept which represents a paradigm shift in our current understanding of T-cell differentiation. In support of this notion are the observations that T-cell priming in murine lung can occur in the absence of lymph nodes and spleens, suggesting a role for DC-T-cell interactions in peripheral tissue (Constant *et al.*, 2002). Moreover, a very recent study demonstrated that T-cell differentiation and activation following bleomycin-induced lung injury may occur locally in the lung (Oh *et al.*, 2011). This is consistent with reports of infiltration and activation of naïve T-cell in tumours (Thompson *et al.*, 2010); such observations raise the intriguing possibility that naïve CD4⁺ T-cell migration, activation and differentiation may additionally occur at sites of epithelial injury following interactions with tissue-resident cells. Under such circumstances, therefore, one may tentatively hypothesise that TSLP-DCs interact with trafficking naïve CD4⁺ T-cells to induce activation and differentiation into effector Th2 cells. Although immunohistochemical analysis of IPF lung does not demonstrate a significant infiltration with naïve T-cells, such assessment is limited by its inherent snap-shot nature, and is intrinsically unable to offer insights into the potential presence of such cells at early stages of disease. The vast majority of patients with IPF present to physicians relatively late, when fibrosis is already well-established and lung function tests demonstrate significant impairment of pulmonary

function (King *et al.*, 2001). Further studies are clearly required to explore these concepts in this disease setting.

It is not possible from the data presented in this thesis to draw any conclusions as to the site of Th2 cell differentiation following bleomycin-induced lung injury. However, one might address this question by examining the phenotype of lung T-cells in this model by FTY720 administration. FTY720 is an agonist of sphingosine-1-phosphate receptor 1, which selectively inhibits the migration of T-cells from lymph nodes (Worbs *et al.*, 2006). If bleomycin-induced T-cell activation and differentiation occurred solely in lymph nodes, one would not expect to observe the predominance of Th2 cells in the lung following bleomycin challenge in FTY720-treated mice, as reported in the current studies. Such studies may be performed in parallel with analyses of phenotypes of T-cells derived from the mediastinal lymph nodes of IPF patients. In addition, useful insights into the importance of lymph nodes as sites of DC-T-cell interactions in the bleomycin model may be further assessed by evaluating T-cell phenotypes in lymphotoxin- α knockout mice (+/- splenectomy to exclude the spleen as a possible site for DC-T-cell interactions); these mice lack lymph nodes and therefore represent a useful tool to determine the importance of DC trafficking to lymph nodes for T-cell priming and subsequent differentiation.

It is important to note that TSLP-DCs co-cultured with naïve CD4 T-cells in a syngeneic system do not induce differentiation of these lymphocytes into Th2 cells, but rather only promote proliferation (Watanabe *et al.*, 2004). Induction of Th2 differentiation *in vitro* is dependent on TSLP-DCs priming naïve T-cells in an antigen-specific manner (Soumelis *et al.*, 2002). Thus, the suggestion that TSLP plays a role in the pathogenesis of IPF implies the presence of a pathogenic antigen. In fact, there is growing evidence that the pathogenicity of T-cells in IPF is indeed antigen-dependent. First, the lymphoid aggregates that have been recently identified in IPF lung are predominantly composed of recently activated and antigen-experienced T-cells (Marchal-Somme *et al.*, 2006). Second, as discussed in **Section 1.6.2.**, the high proportion of circulating CD28-ve T-cells is extremely suggestive of an antigen-dependent T-cell response in IPF (Gilani *et al.*, 2010). Third, abnormal CD4+ T cell clonal expansions, representing cellular immune responses to antigens, have been demonstrated in both serum and BALF of IPF patients (Feghali-Bostwick *et al.*, 2007; Shimizudani *et al.*, 2002). Furthermore, protein extracts from IPF lung have been shown to induce the proliferation of autologous T-cells in a manner suggesting the presence of an intra-pulmonary

antigen capable of activating the immune system. Moreover, repeated cycles of T-cell stimulation with lung extract at 7 – 10 day intervals results in incrementally enhanced proliferation (Feghali-Bostwick *et al.*, 2007), characteristic of responses to antigen.

Is IPF an antigen-driven disease?

The nature of the antigen(s) that drive(s) T-cell activation, differentiation and proliferation in IPF remains unclear. However, there are a number of different scenarios which may result in pathogenetic antigen presentation and subsequent engagement of the adaptive immune system. First, the source of antigen in IPF may be truly foreign and exogenous. The development of hypersensitivity pneumonitis (HP), for instance, is dependent on the inhalation of an environmental antigen which results in an exaggerated non-allergic immune response leading to alveolar inflammation and subsequent fibrosis (Girard *et al.*, 2004). Although considered a separate disease to IPF, it is instructive that the histological pattern of UIP is found in a significant proportion of patients with chronic HP (Churg *et al.*, 2009), suggesting an overlap of pro-fibrotic pathways in these two conditions. Second, the acquisition of somatic mutations may result in the expression of altered protein. Such proteins would then be regarded as “non-self” by the host with subsequent engagement of the adaptive immune response. These mutations may be spontaneous or induced by environmental factors. Moreover, an individual may be further genetically predisposed to the development of such mutations. The most widely recognised environmental factor linking the development of somatic mutations to respiratory disease is cigarette smoking. The acquisition of somatic mutations has been proposed to play a role in the pathogenesis of lung cancer and COPD (Anderson *et al.*, 2003; Roland *et al.*, 1998). As discussed in **Section 1.2.**, cigarette smoking is also a risk factor for the development of IPF (Baumgartner *et al.*, 1997). More recently, cigarette smoking has further been shown to exert a negative influence on both morbidity and mortality of IPF patients (Antoniou *et al.*, 2008). Thus, one may postulate that chronic exposure to an environmental agent, capable of inducing somatic mutations, may result in the expression of a mutated protein that would activate the immune response in a pathological manner. Alternatively, environmentally-induced lung injury may result in the accessibility to the immune system of normally sequestered self-epitopes to which central and peripheral tolerance has never developed. For example, recent studies have demonstrated that cigarette smoking induces the release of proteolytic enzymes

from innate immune cells, liberating elastin fragments which induce an antigen-specific immune response (Lee *et al.*, 2007b). The structure of self-proteins may also be modified by reactive oxygen species (ROS). The ROS-induced modification of amino acids may occur in a number of ways, including the oxidation of sulphhydryl groups on cysteine residues to sulphenic or sulphinic derivatives, inducing protein dimerization (Thannickal *et al.*, 2000). Such modification may then create an epitope to which the host has not previously developed tolerance, and would therefore initiate an adaptive immune response. Cigarette smoke is well recognised to contain reactive oxygen species in both the gas and tar phase (Church *et al.*, 1985), which may serve to modify the structure of proteins in the lung. Very recent studies have demonstrated that chronic oxidative stress, which is associated with increased levels of highly reactive carbonyls in the lung, renders self-proteins immunogenic through carbonyl modification in COPD (Kirkham *et al.*, 2011). Moreover, numerous studies have demonstrated the presence of an increased oxidative burden in IPF lung, irrespective of environmental influences (Jack *et al.*, 1996; Montuschi *et al.*, 1998), characterised by an excess of ROS which overwhelm the host's natural antioxidant defences, including glutathione (Behr *et al.*, 2002). A similar process may therefore occur in IPF; in susceptible individuals, therefore, cigarette smoking may initiate activation of the adaptive immune system, resulting in the inappropriate maturation of self-reactive T-lymphocytes targeted against previously "hidden" antigens. Finally, individuals with IPF may possess an inherent defect in the function of regulatory T-cells (Tregs). These cells are critical to the maintenance of self-tolerance in the periphery (Valencia *et al.*, 2007) and loss of the Treg-mediated suppressive effect on the immune system would permit the inappropriate presentation of self-peptides to immune cells, resulting in T-cell activation. In support of this notion is the recent demonstration of a quantitative and qualitative defect in Tregs from IPF patients (Kotsianidis *et al.*, 2009), similar to that found in other auto-immune diseases, such as rheumatoid arthritis (Ehrenstein *et al.*, 2004).

Is IPF an autoimmune disease?

The concept that IPF may represent an auto-immune disease is also gaining strength. The development of auto-immune pathology is ultimately controlled by genetic and environmental factors which interact to induce host susceptibility to the development of T- and B-cell responses directed against self-peptides.

The generation of pathogenic auto-antibodies in humans is felt to be T-helper cell-dependent, reliant on CD40 / CD40L interactions (Sempowski *et al.*, 1997). It has

been suggested that T-2 cytokines, derived from Th2 cells, play a dominant role in promoting antibody-dependent autoimmune disease (Guo *et al.*, 1999), though this is not a universal finding and is likely to vary from organ to organ (Elson *et al.*, 2000). The role of autoimmunity in the development of fibrosis is currently an area of fertile research and is discussed in more detail in **Section 1.6.2**. Briefly, however, a number of different circulating autoantibodies have been detected in IPF patients (Taille *et al.*, 2010; Yang *et al.*, 2002), though evidence demonstrating causality are lacking. Nonetheless, there remains strong circumstantial evidence in support of a role for pathogenic antibodies directed against self-antigen in IPF. Although the lymphoid aggregates observed in IPF lung are composed predominantly of antigen-experienced T-cells, significant B-cell infiltration is also observed, suggesting the functional interactions between these lymphocytes relating to antigen-presentation (Marchal-Somme *et al.*, 2006). Significant immune-complex deposition has also been reported in IPF lung (Magro *et al.*, 2006) and the highly biased TCR β -chain variable region (TCRBV) repertoire in IPF T-cells (Feghali-Bostwick *et al.*, 2007) supports the notion that the generation of antibodies directed against self-peptides, a T-helper cell-dependent process, contributes to the pathogenesis of IPF. The capacity for TSLP to induce an expansion in the population of previously activated Th2 cells, may therefore potentially promote subsequent T- and B- cell interactions, leading to increased pathogenetic autoantibody generation. However, these notions remain speculative, and further studies are required to determine whether the presence of auto-antibodies in IPF is an epiphenomenon or if they truly contribute to disease pathogenesis.

Much attention has also focused on the potential pathogenetic role of viral infection in the development of IPF. A number of viruses have been implicated in this regard, including cytomegalovirus (Yonemaru *et al.*, 1997) and hepatitis C (Meliconi *et al.*, 1996). More recently, members of the herpes virus family (Tang *et al.*, 2003), including EBV (Sides *et al.*, 2010; Stewart *et al.*, 1999) and human herpesvirus-6 (Pulkkinen *et al.*, 2011), have been implicated in the pathogenesis of IPF. In light of the growing evidence in support of an auto-immune phenomenon in IPF, it is tempting to speculate that the T-cell dependent generation of antibodies generated against self-peptide may occur through “molecular mimicry” following viral infection. This concept proposes that cross-reactive anti-pathogen antibodies, generated following viral infection, bind to self-proteins of the host. These antibody-bound self-proteins may then be processed by DCs, leading to their activation and subsequent functional engagement with T-cells resulting in an oligoclonal expansion of host-

directed T-cells. Such DC activation in the context of increased expression of TSLP following sustained epithelial injury would promote an oligoclonal expansion of Th2 cells, with potentially pro-fibrotic sequelae. There is growing evidence to support the notion that molecular mimicry following EBV infection plays a pathogenetic role in the development of auto-immune diseases such as systemic lupus erythematosus, a condition associated with interstitial lung disease, in humans (Poole *et al.*, 2006). However, one must acknowledge that although a number of antibody targets have been identified in IPF (Dobashi *et al.*, 2000; Taille *et al.*, 2010; Yang *et al.*, 2002), no cross-reactivity has yet been demonstrated to any proteins derived from viruses implicated in the pathogenesis of IPF. Further studies are required to evaluate this concept in this disease context.

It is also important to acknowledge that while the proposed T-cell dependent immunopathology outlined herein is antigen-dependent, disease initiation and progression may be dependent on exposure of the host to multiple antigens. In this regard, molecular mimicry between viral and host antigen may result in the generation of autoreactive T-cells without clinical consequence - autoimmunity without disease - which reflects the need for a critical number of autoreactive T-cells to be generated before disease occurs (Sevilla *et al.*, 2000). In these circumstances, activation of DCs by TSLP at sites of epithelial injury could potentially result in DC-mediated bystander activation of antigen-experienced resident T-cells in the lung, resulting in the expansion of an effector population of Th2 cells specific to a historic, possibly silent, “priming” antigenic stimulus. It is also plausible that this aberrant immunopathology may be further compounded following subsequent viral infections by host heterologous immunity mediated by cross-reactive mechanisms, a phenomenon previously reported in inflammatory diseases of the lung. For instance, acute vaccinia virus (VV) infection of naïve mice results in a severe neutrophilic bronchiolitis with a high degree of mortality. In contrast, VV infection of lymphocytic choriomeningitis virus (LCMV)-immune mice is associated with increased survival, though at the expense of the development of bronchocentric fibrosis, suggesting modulation of the immune response, dependent on previous priming events (Chen *et al.*, 2001; Chen *et al.*, 2003). Interestingly, polarisation of the immune response towards a T-2 phenotype is observed in this model, supporting the notion that heterologous immunity may be influenced by the initial skewing of resident memory T-cells. These exciting concepts require further evaluation in both murine models of lung fibrosis and human disease but may help reconcile a number of seemingly

disparate hypotheses regarding the pathogenesis of IPF, including epithelial injury, aberrant cytokine phenotypes and viral infections.

DC-derived chemokines in IPF

As discussed in **Section 1.4.6.**, TSLP induces the upregulation of OX40L expression by DCs to promote the differentiation of inflammatory Th2 cells. In addition, TSLP promotes the generation of T-2 chemokines, including CCL17 and CCL22 by DCs (Soumelis *et al.*, 2002). These chemokines have previously been shown to be important in the pathogenesis of bleomycin-induced fibrosis by mediating the recruitment of lymphocytes to the lung following injury (Belperio *et al.*, 2004). Moreover, CCL17 has also been demonstrated to be a strong chemoattractant for CCR4-expressing human Th2 lymphocytes in the context of pulmonary inflammation (Kato *et al.*, 2003). The recruitment of T-cells has been demonstrated to be a prerequisite for the transition from an inflammatory response to the development of fibrosis in the bleomycin model (Oikonomou *et al.*, 2006). Thus, increased expression of TSLP, in response to signals received from an injured epithelium, may drive the further recruitment to the lung of immune cells, such as lymphocytes, capable of pro-fibrotic T-2 cytokine generation, along DC-derived chemokine gradients.

In conclusion, my data, together with current evidence in the literature, has led me to propose the hypothesis that in IPF, TSLP-DCs prime T-cells in an antigen-specific manner to induce their differentiation into effector Th2 cells. Although this is likely to occur in locally draining lymph nodes, recent evidence suggests that local activation and differentiation may also be important. Moreover, TSLP-DCs may also promote an expansion of antigen-experienced Th2 memory cells *in situ*, thus playing a key role in the maintenance and polarisation of Th2 cells at sites of injury. The precise nature of the antigen(s) remain(s) unknown though, as has been discussed above, there a number of biologically plausible candidates, reflecting both exogenous and endogenous sources. Finally, it is tempting to speculate that TSLP may further contribute to the pathogenesis of lung fibrosis through the generation of DC-derived chemokine gradients, which may serve to amplify the recruitment of effector Th2 cells to sites of injury. However, recent evidence suggests that TSLP may also act directly on T-cells themselves, and this concept, in the context of lung fibrosis, will now be explored.

4.3.3. Direct effects of TSLP on T-cells in lung fibrosis

There is increasing evidence that TSLP may contribute to the development of a T-2 immune response via direct effects on T-cells (**Section 1.4.7.**). As well as being capable of inducing a programme of differentiation and proliferation of naïve T-cells into Th2 cells (Omori *et al.*, 2007; Rochman *et al.*, 2007), TSLP can enhance TCR-mediated expression of T-2 cytokines by Th2 memory cells (Kitajima *et al.*, 2011). The strong TSLPR immunoreactivity for lymphocytes within ectopic lymphoid aggregates in IPF lung observed in the current studies is therefore of particular interest. The potential importance of T-cells to the pathogenesis of IPF has been discussed in **Section.1.7.** While acknowledging contradictory results in animal models, there is increasing circumstantial evidence supporting their pathogenicity in human disease (Luzina *et al.*, 2008; Pignatti *et al.*, 2006). Human T-cells have been shown to proliferate in response to exposure to TSLP, though this effect is greater in activated cells, in comparison to resting lymphocytes (Rochman *et al.*, 2007). This is consistent with the notion that TSLP expression is upregulated in an inflammatory cytokine milieu which is likely to be associated with T-cell activation. Moreover, T-cells organised in lymphoid aggregates in IPF lung display an activated phenotype, which would render them more susceptible to the effects of TSLP (Marchal-Somme *et al.*, 2006). In addition, TSLP induces upregulation of the IL-2R α receptor chain expression in human T-cells, thereby increasing their sensitivity to low concentrations of IL-2 (Rochman *et al.*, 2007). This is important since Th2 differentiation *in vitro* is dependent on the presence of IL-2, in addition to IL-4 (Cote-Sierra *et al.*, 2004). Recent evidence suggests that IL-2-induced STAT5 activation is critical to maintain accessibility at the DNase hypersensitivity sites of the *Il4* locus, and this collaboration is required for full Th2 differentiation *in vitro* (Zhu *et al.*, 2003). Thus, a direct effect of TSLP on T-cells to upregulate expression of IL2R α would offer a further mechanism for amplifying the development of a T-2 immune response, by increasing their sensitivity to IL-2. The strong TSLPR immunoreactivity for lymphocytes is therefore compatible with the hypothesis that TSLP may exert direct effects on lymphocytes in the fibrotic lung to induce and maintain a functioning Th2 cell population.

Does TSLP influence Treg function?

The studies presented in this thesis did not explore the recently proposed concept that Tregs may play a role in the pathogenesis of IPF. Analysis of serum and BALF specimens from IPF patients revealed a deficit in Treg number and function

(Kotsianidis *et al.*, 2009). These cells are critical for the control of immunological tolerance, and impairment of their function is associated with the development of autoimmune conditions such as rheumatoid arthritis (Chavele *et al.*, 2011), which is associated with a UIP histological pattern of lung fibrosis. However, during the course of this PhD, *in vitro* studies demonstrated that TSLP directly impairs the immunosuppressive capability of Tregs (Nguyen *et al.*) and as well as Treg development (Lei *et al.*, 2011). One might speculate, therefore, that locally-derived TSLP may serve to impair Treg function in the lung, thus attenuating the immunosuppressive influence of this sub-population of T cells. Indeed, Tregs isolated from IPF lung display a deficit in suppressing T-2 cytokine expression *in vitro* (Kotsianidis *et al.*, 2009), which is consistent with previous studies demonstrating that mice deficient in Tregs display increased airway hyper-responsiveness in models of airway inflammation (Doganci *et al.*, 2005) – this effect is reversed, together with suppression of T-2 cytokine expression, by the adoptive transfer of Tregs (Kearley *et al.*, 2005). Furthermore, the absence of Tregs has been demonstrated to promote a fundamental T-2 bias in mice (Wan *et al.*, 2007) suggesting that TSLP-induced defective Treg function may contribute to polarisation of the immune response towards a T-2 phenotype by ineffective suppression of existing T-cell function. However, the relationship between TSLP and Treg function is far from clear, as other groups have reported that TSLP-DCs promote the development of Tregs *in vitro* (Watanabe *et al.*, 2005). It is likely that the influence of TSLP on Treg development and/or function is dependent on the presence or absence of an associated inflammatory microenvironment, and further studies are required to evaluate this relationship in IPF.

Is there a role for Th17 cells in IPF?

Th17 cells represent a novel subset of T-lymphocytes which have been demonstrated to play a key role in inflammation associated with neutrophil infiltration. The Th17 response in the lung has been most extensively investigated following infectious injury (Korn *et al.*, 2009), though recent studies have also demonstrated its importance in immune cell recruitment following non-infectious injury (Lo Re *et al.*, 2010). The prophylactic administration of anti-IL17R antibodies attenuates bleomycin-induced fibrosis, reflecting an impairment of immune cell recruitment in the first week after injury. Interestingly, these studies also demonstrated that differentiation of Th17 cells occurred in the lung following injury, and not in local lymph nodes, suggesting the development of a tissue

microenvironment conducive to influencing T-cell behaviour *in situ* (Oh *et al.*, 2011). In light of recent evidence suggesting a role for TSLP in the development of such cells (Tanaka *et al.*, 2009), one may tentatively speculate that locally recruited T-cells to sites of injury may be exposed to a Th17-promoting microenvironment characterised by increased TSLP expression. Their subsequent differentiation into this subset would promote the recruitment of effector cells, such as neutrophils during the inflammatory phase of bleomycin-induced injury, the actions of which contribute to fibrogenesis (Chua *et al.*, 2007). However, much more work is required to draw any firm conclusions regarding the role of this cytokine in Th17 cell differentiation, let alone the contribution of this T-cell subset to the pathogenesis of lung fibrosis.

In conclusion, therefore, the data presented in this thesis support the notion TSLP may contribute to the pathogenesis of IPF by inducing and maintaining a population of effector Th2 cells, capable of elaborating pro-fibrotic T-2 cytokines at sites of epithelial injury. Having demonstrated potential human disease relevance for TSLP with respect to the pathogenesis of IPF, the potential contributory role of TSLP to the development of lung fibrosis was explored in the bleomycin model, and these findings will be discussed in **Section 4.7**. However, the present studies, to the best of my knowledge, represent the first to highlight the fibroblast as a potential cellular source of this cytokine in the lung, supporting the notion that this cell possesses important immunoregulatory functions, and these concepts will now be discussed in more detail.

4.4. TSLP production by structural lung cells *in vitro*

As discussed in **Section 4.1.**, strong immunoreactivity for TSLP was observed for epithelial cells and fibroblasts in fibrotic lung disease. Immunohistochemical techniques do not, however, offer definitive evidence of cellular expression, but instead suggest cellular localisation. To corroborate these findings, the expression of TSLP by these cell types was evaluated *in vitro*.

4.4.1. TSLP expression by alveolar epithelial cells

Despite strong immunoreactivity for TSLP in the fibrotic human lung, we were unable to demonstrate that TSLP was secreted by either A549 epithelial cells or primary type II epithelial cells freshly isolated from donor lung. There are several potential explanations for these findings. First, our *in vitro* studies were performed using cells derived from non-fibrotic human lung tissue or from young donors with

no obvious lung pathology. TSLP immunoreactivity for alveolar epithelial cells in control human lung was relatively weak and occasional, in contrast to the strong signal observed in fibrotic disease. Therefore, the lack of baseline or inducible expression may reflect a change in the transcriptional profile of reparative hyperplastic epithelial cells as compared to normal epithelium, reflecting chronic and sustained injury. This phenomenon is increasingly recognised in a number of other cell types, including mesenchymal cells, and will be discussed in more detail in **Section 4.4.2**. Ideally, TSLP expression would have been further assessed in AECs derived from fibrotic lung. However, the preparation of primary type II AECs requires a large amount of fibrotic tissue (>150 g), which is much greater than that obtained from lung biopsies. One is therefore reliant on explanted lung from IPF patients undergoing transplantation, and the relative lack of such patients precluded further studies in primary type II alveolar epithelial cells during the course of this PhD. Although the lung epithelial cell line, A549, is a useful tool to initiate investigations into epithelial cell biology, it cannot be relied on to provide definitive evidence of functional and biologically relevant responses. Moreover, A549 cells are well-known to display functional variability in *in vitro* assessments, which may explain the discrepancies between the current findings and reports of inducible TSLP expression by this cell line (Lee *et al.*, 2007a). In light of these findings, the focus of the current studies shifted to evaluate baseline and inducible expression of TSLP by primary adult human lung fibroblasts (pHLFs), also identified by immunohistochemical techniques as a possible novel cellular source of TSLP in the context of IPF as well as other respiratory conditions.

4.4.2. TSLP expression by primary adult human lung fibroblasts

Although lung fibroblasts represent a key effector cell common to airway and parenchymal lung disease, their ability to express TSLP has, until recently, not been formally evaluated. This is in contrast to fibroblasts in other organ pathologies, including nasal polyp and synovial fibroblasts. (Koyama *et al.*, 2007; Nonaka *et al.*, 2010). In addition, a role for fibroblast-derived TSLP has been postulated in the development of a GATA-3^{hi} lymphoid infiltrate in pancreatic cancer which is associated with a poor prognosis (De Monte *et al.*).

The studies presented in this thesis demonstrate that lung fibroblasts are capable of elaborating TSLP in response to stimulation with TNF- α in a time- and concentration-dependent manner; this is most likely to occur via transcription of the long splice variant of the *Tslp* gene. The potential clinical relevance of TSLP

variants is highlighted by studies demonstrating that a single nucleotide polymorphism (SNP) in the promoter region of the long splice variant, which is associated with enhanced TSLP expression *in vitro*, (Harada *et al.*, 2009) is significantly associated with disease susceptibility in asthma (Harada *et al.*, 2011). However, the relationship of this SNP to lung fibrosis has not been examined; such studies may offer further insights into the potential importance of this cytokine in the pathogenesis of IPF.

In the present studies, TNF- α was found to induce TSLP expression at both the protein and mRNA levels, but the magnitude of the mRNA response was not mirrored by such an impressive induction at the protein level. Such discrepancy between the abundance of protein and mRNA has been documented previously in other systems (Garrison *et al.*, 2010; Ralph *et al.*, 2005) and represents low translational efficiency for this gene. It is possible that TSLP protein synthesis in pHLFs is tightly regulated by post-translational protein modification to ensure that extracellular functional TSLP concentrations are maintained within a small effective window. Elevated levels of circulating TSLP during murine neonatal haematopoiesis has been shown to lead to a significant expansion of B cell precursor populations, resulting in a lethal infiltrative B cell lymphoproliferative disorder (Demehri *et al.*, 2008), suggesting that TSLP may have severe systemic effects at high concentrations. Moreover, systemic overexpression of TSLP has also been reported to result in a severe global inflammatory response secondary to cryoglobulinaemia (Taneda *et al.*, 2001), further highlighting the importance of the need for tight local regulation of this cytokine. In addition, this discrepancy may be explained by post-transcriptional modification of mRNA-binding regulatory factors. In this regard, it is possible that interactions between RNA binding proteins and cognate cis-elements in untranslated regions of TSLP mRNA may influence the translation of TSLP mRNA.

In summary, the data presented in this thesis support the notion that TNF- α , a danger signal reflecting tissue injury, is capable of modulating the nature of the immune response by inducing TSLP production by pHLFs. Moreover, they highlight the potential for fibroblasts to function in an immunoregulatory capacity, promoting the switch from innate to adaptive immunity via elaboration of this key T-2 polarising cytokine. There is increasing recognition of the ability of mesenchymal cells to influence immune responses, and evidence suggests that such interactions may be mediated by direct cell-cell interactions or indirectly, in particular by chemokines.

Direct regulation of haematopoietic cells by fibroblasts has previously been reported to be mediated by CD40-CD40L interactions (Sempowski *et al.*, 1997). Such a bi-directional relationship would serve to promote activation of CD40 expressing fibroblasts leading to the generation of chemokine gradients, serving to enhance further recruitment of immune cells. The generation of such stromally-derived chemokine gradients will be discussed in more detail in **Section 4.5.1**. Importantly, recent studies have suggested that chemokines also possess direct immune-modulatory effects (Luther *et al.*, 2001), and this concept will also be discussed in **Section 4.5.1**. In addition, there exists strong evidence that fibroblasts comprise a heterogeneous population, with *in vitro* functional differences in migratory capacity and ECM production dependent on the site of isolation (Fries *et al.*, 1994). In fact, such differences appear dependent upon the presence or absence of inflammation. Global gene expression studies have demonstrated that the transcriptional profile of fibroblasts may be modified by inflammatory stimuli, such as TNF- α , towards a more immuno-centric phenotype (Parsonage *et al.*, 2003). For instance, murine lung fibroblasts derived from Th2-induced granuloma spontaneously elaborate significantly higher quantities of the immunomodulatory chemokine, CCL2, than their Th1-derived counterparts (Hogaboam *et al.*, 1999). It has been postulated that fibroblasts may be broadly divided into possessing peripheral or lymphoid characteristics, and TNF- α seems to promote a more lymphoid transcriptional profile (Parsonage *et al.*, 2003). In this regard, it is interesting to reflect on the presence of lymphoid aggregates in IPF lung tissue, a condition characterised by increased expression of TNF- α . The presence of such tertiary lymphoid tissue (Ruddle, 1999) is well described in chronic inflammatory conditions, such as auto-immune thyroid disease (Armengol *et al.*, 2003) and rheumatoid arthritis where the continued recruitment of inflammatory cells is associated with persistent fibroblast activation (Buckley, 2003). Ideally, interactions between recruited immune effector cells and stromal cells at sites of injury would ultimately result in resolution of the inflammatory response, in part due to the withdrawal of stromally-derived survival or chemokine factors (Buckley *et al.*, 2001). An inflammation-induced change in the nature of the fibroblast towards a lymphoid phenotype, with associated changes in chemokine/adhesion molecule expression, may therefore promote the development of stroma more akin to that found in classical lymphoid organs. In turn, this would be capable of supporting retained effector lymphocytes within a tertiary lymphoid structure, promoting a persistent immune response.

4.4.3. Delineation of the signalling pathways involved in TNF- α induced TSLP production by pHLFs

Signal transduction plays a critical role in converting extracellular stimuli into appropriate cellular responses. Understanding the mechanisms by which extracellular stimuli modify the function of cells not only provides valuable insight into the pathogenesis of disease, but may also lead to the identification of novel therapeutic targets for pharmacological intervention. A number of tools are available to delineate signalling pathways. The present studies employed both pharmacological tools and genetic approaches to identify those pathways important in mediating TNF- α -induced expression of TSLP in pHLFs.

During the course of these studies, the potential roles of the transcription factors, NF κ B and AP-1, as well as members of the MAPK family, were evaluated. Despite engagement with their respective targets, as demonstrated by Western blotting, inhibitors of NF κ B, p38 and ERK1/2 did not exert a concentration-dependent attenuation of TNF- α -induced TSLP protein release, enabling the confident exclusion of these signalling pathways in mediating this response. Any inhibitory effects observed are therefore likely to represent off-target effects of the inhibitors used. These findings are in contrast to previous reports demonstrating the importance of these pathways in mediating TSLP expression in lung epithelial lines, bronchial epithelial cells and HASMCs. However, it is plausible that different signalling pathways predominate in different cell types. In contrast, a central role for the JNK/c-Jun signalling pathway in mediating TNF- α -induced TSLP expression was identified, and these findings will now be discussed in more detail.

4.4.3.1. Role of JNK pathway

JNK is a member of the MAPK family and, as discussed in **Section 1.4.3.1.**, only JNK1 and JNK2 are relevant to respiratory disease. JNK is the enzyme responsible for phosphorylating critical serine residues in the N-terminus of c-Jun, a component of AP-1. Pharmacological inhibition of the JNK pathway has been shown to reduce the degree of eosinophilic inflammation and airway hyper-responsiveness in a murine model of asthma (Nath *et al.*, 2005), and JNK1 knockout (JNK1^{-/-}) mice are protected from bleomycin induced lung fibrosis (Alcorn *et al.*, 2009).

There is good evidence to support a role for AP-1, a major target of JNK, in mediating the expression of TSLP (**Section 1.4.3.2.**). To investigate the role of JNK MAPK, and its target transcription factor, AP-1, the effect of two pharmacologically

distinct inhibitors of JNK activity, SP600125 and TI-JIP, on TNF- α -induced TSLP expression was examined. SP600125 is a reversible ATP competitive inhibitor of JNK activity. TI-JIP is a peptide corresponding to the amino acid sequence of the JNK interacting protein-1 scaffold protein that potently inhibits JNK activity in a manner that is non-competitive for ATP (Barr *et al.*, 2004). In the present studies, significant concentration-dependent attenuation of TNF- α -induced TSLP protein release was observed using both inhibitors. Moreover, TNF- α -induced increases in TSLP mRNA levels are inhibited by SP600125, supporting the notion that *Tslp* gene transcription in pHLFs is dependent on JNK, most likely via activation of AP-1. Indeed, TNF- α -induced TSLP protein release was also significantly inhibited in the presence of the AP-1 inhibitor, curcumin. However, although pharmacological inhibition of signalling pathway components is a useful tool, non-specific effects of inhibitors cannot be excluded completely. RNA interference was therefore employed to confirm these findings.

RNA interference (RNAi) is a highly conserved genetic surveillance mechanism which permits post-transcriptional down-regulation of target genes in a sequence-specific manner. Briefly, the RNAi pathway is initiated by Dicer, an endoribonuclease which cleaves long dsRNA molecules into short interfering RNA molecules (siRNA). This dsRNA may be exogenous, such as viral, or endogenous, such as microRNAs (miRNA) which are genomically coded non-coding mRNA molecules. It is increasingly recognised that such miRNAs play critical roles in organogenesis via post-transcriptional regulation of target gene expression (Stefani *et al.*, 2008) and dysregulation of miRNA expression is increasingly implicated in a number of disease processes, including IPF (Pandit *et al.*, 2010). Following unwinding of the siRNA molecule into 2 single-stranded RNA molecules in an ATP-dependent manner, the guide strand is then incorporated into a multi-protein RNA-silencing complex (RISC). This anti-sense guide strand then directs the entire complex to an mRNA molecule bearing a complementary sequence, the target mRNA, which is subsequently degraded by catalytic components of RISC, thus preventing its translation. Such post-transcriptional gene silencing ultimately leads to a reduction in the functional levels of target protein levels within the cell.

Experimental RNAi exploits this mechanism to allow specific knock-down of protein expression in cells, enabling their importance to particular pathways to be evaluated. However, a significant problem of introducing long dsRNA molecules into eukaryotic cells is the initiation of an anti-viral apoptotic pathway, leading to cell

loss. This issue may be circumvented by the transfection of siRNA, specific for the target mRNA of interest. The introduction of siRNA into cells may be achieved by direct transfection or by using plasmid or viral vectors. In the current studies, transfection was achieved using a “Gemini reagent”, a kind gift from GSK, which has previously been used in the host laboratory to good effect.

The role of c-Jun in mediating TNF- α -induced TSLP expression was confirmed by transfecting pHLFs with siRNA targeted against c-Jun mRNA. Successful knock-down of c-Jun protein expression, under conditions of TNF- α exposure, was confirmed by Western blotting. This knock-down led to a significant inhibition of the TNF- α -induced TSLP signal, confirming the importance of the JNK / c-Jun pathway in regulating this response.

During the course of my studies, upregulation of TSLP expression at the protein level was reported for normal human lung fibroblasts (NHLFs) following exposure to TNF- α (Futamura *et al.*, 2010). This effect was enhanced by β -agonists and c-AMP elevating agents and attenuated by steroids in a cAMP-independent manner (Futamura *et al.*, 2010). However, the signalling mechanisms underlying TNF- α -mediated TSLP expression were not further evaluated in these studies. β -agonists act on G-protein coupled receptors (GPCRs), a major group of transmembrane receptors responsible for detecting extracellular signals. GPCRs, particularly those coupled to Gai and Gaq families of proteins are strongly linked to the activation of JNK. GPCRs coupled to Gas, including the β -agonist receptor, induce the generation of cAMP. In an effort to reconcile these observations, it could be hypothesized that cAMP positively regulates JNK activity. Indeed, this has previously been demonstrated in a number of different cell types (Kanno *et al.*, 2004; Yamauchi *et al.*, 2001). However, this was not a universal finding, and there are reports of JNK activity being inhibited by elevated cAMP levels (Zhang *et al.*, 2008). Therefore, it is possible that the regulation of JNK activity by cAMP is cell-specific.

TNF- α -induced TSLP expression in fibroblasts has also been demonstrated to be inhibited by fluticasone (Futamura *et al.*, 2010). Glucocorticoids (GCs), including fluticasone, exert the vast majority of their actions by binding to intracellular glucocorticoid receptors (GRs). The most well-described mechanism through which they influence gene transcription is via the bindings of these receptors to responsive elements in the regulatory sequences of target genes. However, cross-talk between GRs and other transcription factors is also recognised, and GR antagonism of NF κ B

and AP-1 is thought to underlie the anti-inflammatory actions of steroids (Herrlich, 2001). GRs may interfere with AP-1 directly, preventing effective interactions between AP-1 and the transcriptional machinery. However, GCs have also been demonstrated to repress AP-1-mediated transcription of genes by inhibiting phosphorylation of JNK (Bruna *et al.*, 2003). Thus inhibition of TNF- α -induced TSLP expression in fibroblasts by glucocorticoids is consistent with their known ability to inhibit AP-1 activity. These observations are therefore compatible with the present studies highlighting the importance of the JNK / AP-1 signalling pathway in mediating the expression of TSLP in pHLFs.

4.5. pHLFs express a functional TSLP receptor signalling axis

As discussed in **Section 4.2.**, the current studies demonstrated immunolocalisation of TSLPR to fibroblasts in both murine and human fibrotic lung, suggesting that this cell type may represent a novel cellular target for TSLP. These findings are consistent with studies published during the course of this thesis which showed that mesenchymal cells, such as human airway smooth muscle cells, also express a functional TSLP receptor (Shan *et al.*, 2010). The expression of both components of the TSLP receptor was therefore confirmed at both the mRNA and protein level in pHLFs before functionality was examined *in vitro*. In light of the increasing importance attached to the generation of stromally-derived chemokine gradients in inflammatory disease, chemokine expression by pHLFs following exposure to TSLP was evaluated. These studies demonstrated that TSLP upregulates the expression of CCL2 in pHLFs in a time- and concentration-dependent manner. The implications of these observations will now be discussed.

4.5.1. TSLP induces the expression of CCL2 in pHLFs

CCL2 is a member of the CC chemokine family with a wide range of cellular sources including lung fibroblasts (Deng *et al.*, 2008). There is strong evidence suggesting a pathogenic role for this chemokine in lung fibrosis via a number of potential mechanisms. First, CCL2 is a potent chemoattractant for immune cells, including T-cells and dendritic cells (Carr *et al.*, 1994; Xu *et al.*, 1996). The present studies showed that TSLP is capable of inducing chemotaxis of immune cells in a CCL2-dependent manner, suggesting that TSLP may represent an important mediator of immune cell trafficking. In fact, the expression of chemokines by structural cells is key to effective DC trafficking murine lung. In particular, the interstitial location of fibroblasts suggests that, in addition to matrix deposition, they may serve to

influence immune cell migration out of the vasculature. Very recent studies have demonstrated the importance of fibroblast-derived CCL2 in mediating DC trafficking in lung inflammation *in vivo* (Kitamura *et al.*, 2011). Moreover, fibroblasts exposed to TNF- α are capable of promoting DC migration *in vitro* via the generation of secondary mediators (Saalbach *et al.*, 2010), suggesting that these cells are capable of actively participating in the development of an immune response at sites of injury. This generation of a stromally-derived chemokine gradient is increasingly recognised as crucial to the accumulation, differentiation and survival of immune cells in non-lymphoid organs (Buckley *et al.*, 2001). Conversely, withdrawal of such signals has been postulated to be important in the resolution of inflammation. Increased expression of chemokines, including CCL19, CCL22 and CXCL12 has been reported in IPF lung, localising to fibroblasts and epithelial cells. CCL19, in particular, has been reported to be an important chemoattractant for DCs (Scandella *et al.*, 2004), while the major function of CCL22 is to promote recruitment of activated T-cells, particularly Th2 cells (Andrew *et al.*, 1998). CXCL12, on the other hand, is important for the successful trans-endothelial migration of DCs (de la Rosa *et al.*, 2003). The close structural proximity of immune cells to stromal cells expressing these chemokines in IPF is suggestive of a functional interaction, as is found in chronic inflammatory conditions, such as rheumatoid arthritis. These conditions are characterised by the persistent recruitment of immune cells ultimately resulting in the development of tertiary lymphoid structures within a stable but disordered matrix, such as has been recently reported in IPF (Marchal-Somme *et al.*, 2006). The present findings suggest an additional novel role for TSLP in the generation of stromally-derived chemokine, particularly CCL2, serving to attract effector immune cells, such as monocytes/macrophages, to sites of injury. Further *in vitro* studies are required to examine the role of TSLP in promoting T-cell and DC chemotaxis: subsequent functional interactions between these cell types, in the presence of fibroblast-derived TSLP, would serve to promote a local T-2 dominated cytokine milieu. It must be noted, however, that conditioned media from pHLFs exposed to TSLP retained some chemotactic properties for human monocytes despite neutralisation of CCL2. These data suggest the elaboration of further chemokines by pHLFs in response to stimulation with TSLP. The current work attempted to identify the nature of these additional mediators using the MSD platform to examine TSLP-induced chemokine protein release. However, this was not fruitful and requires further studies.

As suggested above, the mechanisms by which CCL2 promotes fibrosis are likely to extend beyond its chemoattractant properties and include upregulation of TGF- β expression and possibly that of the IL-13 receptor chain IL-13R α 1/2 by fibroblasts (Gharaee-Kermani *et al.*, 1996; Murray *et al.*, 2008). In light of the current findings, I would propose that TSLP may represent an alternative mediator of CCL2 expression in pHLFs, which would serve to promote the fibrotic response.

In addition to the effects described above, it is increasingly recognised that chemokines possesses important immunoregulatory capabilities, including the regulation of T-cell differentiation. Exposure of T-cells to CCL2 *in vitro* results in increased T-2 cytokine expression (Karpus *et al.*, 1997), an effect which is enhanced in recently-activated or memory T-cells. This is consistent with the observation that CCR2 is not expressed by naïve T-cells, but rather on recently activated CD4⁺ cells (Loetscher *et al.*, 1996; Qin *et al.*, 1996). As previously discussed, the majority of T-cells organised within lymphoid aggregates in IPF display an activated phenotype, thus rendering them susceptible to further modulation by CCL2. In addition, neutralisation of fibroblast-derived CCL2 has been shown to attenuate CD4⁺ IL-4 production, with a concomitant increase in IFN γ expression (Hogaboam *et al.*, 1998), suggesting that CCL2 is capable of modulating CD4⁺ T-cell behaviour directly. However, CCL2 may promote the development of Th2 cells via the generation of a Th2-permissive milieu, much like the effects of TSLP on DCs. The differentiation of Th2 cells requires an IL-12_{lo} microenvironment, and CCL2 has been demonstrated to downregulate the expression of this T-1 polarising cytokine by activated monocytes *in vitro* (Chensue *et al.*, 1996). These *in vitro* findings have been translated to animal models of disease, where CCL2 knock-out mice are unable to mount a protective T-2 immune response to *Leishmania* major infection, despite normal lymphocyte trafficking (Gu *et al.*, 2000).

4.5.2. TSLP-induced CCL2 expression in pHLFs is STAT3-dependent

The present studies identified STAT3 as the major signalling pathway involved in mediating CCL2 expression in pHLFs exposed to TSLP. Critical to STAT activation is the dimerization of two STAT monomers. This dimerization is dependent upon the binding of the -SH2 domain of one monomer to a Pro-pTyr-Leu-Lys-Thr-Lys sequence on the complementary monomer. However, prior to this, STAT monomers must bind to tyrosine motifs on the receptor which have been phosphorylated by receptor-associated JAKs. S3I-201 is a non-peptide small molecule which targets the -SH2 domain and prevents STAT3 from binding to the pTyr residues of the

receptor, thus blocking *de novo* phosphorylation by receptor-associated JAKs (Siddiquee *et al.*, 2007). The relatively high concentrations of S3I-201 used in the current studies is comparable to previous reports (Pang *et al.*, 2010). Nonetheless, it was important to confirm the role of STAT3 in mediating TSLP-induced CCL2 expression using a genetic approach. These studies showed that knockdown of STAT3 expression using siRNA attenuated TSLP-induced CCL2 expression.

The current findings are consistent with very recent reports published during the course of these PhD studies showing that ligation of the TSLP receptor also results in STAT3 activation in other mesenchymal cells, such as airway smooth muscle cells, with subsequent upregulation of chemokine expression (Shan *et al.*, 2010). Activation of STAT5 has also been implicated in mediating the cellular effects of TSLP in haematopoietic cells (Isaksen *et al.*, 1999). However, STAT5 was not found to be phosphorylated in pHLFs following TSLP exposure. These differences in STAT phosphorylation may reflect mesenchymal cell-specific signalling events downstream of TSLP receptor ligation, as TSLP does not induce STAT5 phosphorylation in HASMCs either (Shan *et al.*, 2010). The present reported findings are also consistent with previous observations demonstrating that STAT3 activation is important in mediating CCL2 expression in a variety of cell types (Kujawski *et al.*, 2008; Lee *et al.*, 2011; Schroer *et al.*, 2011). Moreover, using a combination of oligonucleotide gene arrays and qRT-PCR, a group of genes involved in chemotaxis and wound healing has recently been identified whose transcription is mediated by STAT3, including CCL2 (Dauer *et al.*, 2005).

In conclusion, the findings discussed in this section show for the first time that lung fibroblasts are capable of responding to TSLP, further highlighting the potential immunoregulatory importance of these cells. It is tempting to speculate that TSLP acts in an autocrine manner on fibroblasts to upregulate CCL2 expression, and that aside from its pro-fibrotic effects, CCL2 might promote the recruitment of dendritic cells and T-cells, in addition to macrophages. The well-described T-2 promoting properties of TSLP may then be further amplified by the immunomodulatory effects of CCL2 on recruited naïve, recently activated and/or memory T-cells. However, it is clear that additional work is required to definitively assign a mechanistic role for TSLP-induced CCL2, derived from lung fibroblasts, in fibroproliferative lung disease.

4.6. Role of TSLP in modulating innate and adaptive immune cell populations following bleomycin-induced lung injury

The role of TSLP in the development of atopic lung disease is well described (Zhou *et al.*, 2005). Such conditions are characterised by increased expression of T-2 cytokines. However, such an immune phenotype is not exclusive to atopic disease and may develop in the absence of allergy. This aspect of TSLP biology has not previously been examined in the lung, and so the next logical step of this research study was to explore the functional role of this cytokine in an animal model of fibrosis. As discussed in **Section 1.3.1.**, the bleomycin model of lung injury was chosen for these exploratory studies.

4.6.1. *TSLP mediates bleomycin-induced dendritic cell activation but not recruitment to the lung*

A central theme of this thesis has been that, following epithelial injury, activation of dendritic cells by TSLP promotes a pro-fibrotic immune response. The data discussed in **Section 4.1.** established potential human disease relevance for this hypothesis by demonstrating increased TSLP immunoreactivity for cells in close proximity to dendritic cells, a major cellular target for this cytokine. During the course of this PhD, bleomycin-induced lung injury was reported to lead to an accumulation of dendritic cells and increased local expression of DC-attracting chemokines, including CCL2 (Bantsimba-Malanda *et al.*, 2010). Further analysis of these lung DCs revealed that they were of a mature and activated phenotype, as evidenced by increased cell surface expression of CD86. The nature of bleomycin-induced lung injury is felt to reflect DNA scission in epithelial cells (Harrison *et al.*, 1989), with subsequent apoptosis. This change in alveolar epithelial cell phenotype is well recognised in IPF (Kuwano *et al.*, 1996). These observations are compatible with the notion that dendritic cells may be activated by danger signals as previously discussed in **Section 4.3.2.** Such signals, for example uric acid and eATP which have both been implicated in the pathogenesis of IPF (Gasse *et al.*, 2009; Riteau *et al.*, 2010), may be directly released from injured cells. However, DC maturation may also be mediated by heparin sulphate proteoglycans, which are important mediators of fibroblast-matrix interactions (Johnson *et al.*, 2002). The mediators of DC activation following bleomycin-induced lung injury, however, remain unknown. In light of the T-2 polarising phenotype of TSLP-DCs, and the evidence that this model is associated with a T-2 immune response, this PhD examined the hypothesis that lung DC activation following bleomycin challenge is mediated by TSLP.

In the first instance, the present studies demonstrated that lung injury initiated by a single instillation of bleomycin via the oropharyngeal route results in an accumulation of lung DCs which continues into the fibrotic phase of this model. These observations are consistent with previous reports that local expression of chemokines known to attract DCs, including CCL19 and CXCL12 (de la Rosa *et al.*, 2003; Scandella *et al.*, 2004) is upregulated from day 14 post bleomycin challenge. Moreover, they are compatible with the presence of both immature and mature DCs in IPF lung, suggesting continued recruitment in the presence of established fibrosis (Marchal-Somme *et al.*, 2007b; Marchal-Somme *et al.*, 2006).

I next examined the role of TSLP in promoting DC recruitment in response to bleomycin-induced lung injury, using a neutralising anti-TSLP antibody (a kind gift from GSK). Previous animal studies designed to examine the role of TSLP in inflammatory lung disease have employed a variety of techniques, including the use of *Tslpr*^{-/-} mice (Zhou *et al.*, 2005) and the administration of a blocking anti-TSLPR (Shi *et al.*, 2008) or neutralising anti-TSLP antibody (Li *et al.*, 2010). For the current studies, antibody-mediated neutralisation was chosen to block TSLP signalling *in vivo*, since we did not have access to *Tslp* or *Tslpr*^{-/-} mice and were moreover keen to avoid the potential development of compensatory immune mechanisms associated with knock-out mice. The dosing regime and route of administration of the anti-TSLP antibody, 28F12, was based on detailed pharmacokinetic data provided by GSK. Serum samples were taken from mice during the evolution of the bleomycin response to assay antibody concentrations. The efficacy of 28F12 in neutralising TSLP activity *in vitro* was also examined prior to the *in vivo* studies. A concentration-dependent inhibition of TSLP-induced CCL2 protein release by primary murine lung fibroblasts (pMLFs) was observed *in vitro*, with complete inhibition of the CCL2 signal observed at a concentration of 30 ng/ml. Serum concentrations of 28F12 achieved with the outlined dosing schedule were in the range of 100 µg/ml (data presented in **Appendix A1**). These data provided a high degree of confidence that adequate neutralisation of TSLP activity could be achieved.

The chemoattractive properties of TSLP have not been extensively studied. DC migration involves a complex interplay between cell-intrinsic and extrinsic factors. Current evidence suggests that danger signals may induce the upregulation of chemokine receptors on DCs, which are then more receptive to chemokine gradients generated within inflamed tissue [reviewed in (Banchereau *et al.*, 2000)].

During the course of this PhD, TSLP was reported to act as a DC chemoattractant *in vitro* (Fernandez *et al.*, 2011). These findings support a previous observation that anti-TSLPR administration in a murine model of asthma is associated with reduced recruitment of DCs to airways (Shi *et al.*, 2008). In the present studies, neutralisation of TSLP activity did not inhibit the bleomycin-induced accumulation of DCs at day 10 or 28 following lung injury. One plausible explanation to reconcile these findings might be that the role of TSLP as a DC chemoattractant is injury- and context-dependent. In the bleomycin model, DC chemotaxis may be governed by the generation of TSLP-independent chemokine gradients, in contrast to airway disease. Furthermore, it is possible that TSLP-dependent chemotaxis is important for DC migration to lymph nodes, rather than directly from the vasculature to the lung parenchyma, as has been postulated to occur in the bleomycin-induced lung injury and in IPF (Bantsimba-Malanda *et al.*, 2010; Marchal-Somme *et al.*, 2007b).

The current studies confirmed that bleomycin-induced injury is associated with a higher proportion of lung DCs exhibiting an activated phenotype (CD86+) at day 10 post-injury. In contrast, this model of lung injury is not associated with splenic DC activation suggesting that bleomycin challenge is not associated with a systemic immune response, but rather one which is limited to the site of epithelial injury. The observation that lung DC activation following bleomycin-induced lung injury is inhibited in mice administered an anti-TSLP antibody provides strong proof-of-concept that TSLP plays a non-redundant role in mediating local DC maturation during the inflammatory phase of this non-allergen driven model of lung injury. These findings, therefore, extend the role of TSLP as a signal for DC activation to inflammatory conditions beyond allergen-driven inflammation, and identify for the first time a mediator involved in DC activation in the bleomycin model. As discussed in **Section 1.4.5.**, activation of DCs is critical to their subsequent interactions with T-cells. The upregulation of co-stimulatory cell surface proteins, such as CD86, are critical for the activation and differentiation of naïve T-cells into Th2 cells (van Rijt *et al.*, 2004). Indeed, blocking CD86 co-stimulation inhibits reactivation of Th2 memory cells *in vivo* (Keane-Myers *et al.*, 1997). Given the importance of CD86-expressing DCs in T-2 polarisation, and in light of the additional unique T-2 polarising capabilities of TSLP-DCs, it is tempting to speculate that inhibition of TSLP-induced DC activation would inhibit the development of a T-2 immune response in this model. This inhibition may be further compounded by the prevention of direct effects of TSLP on naïve or memory T-cells.

In contrast, it is not currently possible to draw any conclusions regarding the role of TSLP in promoting DC maturation during the fibrotic phase of this model (14-28 days post injury). By this time point, the vast majority of lung DCs examined in all mouse groups were CD86+, with no discernible differences observed between the saline-treated and bleomycin-challenged mice. Over the course of 28 days, each mouse received 8 doses of rat IgG, in contrast to 3 doses during the course of 10 days. The apparent global activation of DCs seen in all groups of mice receiving such dosing may reflect an effect of the interventional rat IgG, in inducing DC maturation. Although, the rat antibodies used in the current studies were designed to be minimally immunogenic in mice, they nonetheless represent foreign protein, and therefore may induce a humoral immune response. DCs are well recognised to express receptors for the Fc domain of immunoglobulins, including FcγR (Sallusto *et al.*, 1994), which binds IgG. Moreover, previous studies have demonstrated that FcγR-mediated internalisation of immune complexes by DCs, consisting of circulating antibody bound to foreign antigen, is associated with DC activation (Regnault *et al.*, 1999). The generation of murine IgG to foreign antigen has been demonstrated to occur within 1-2 weeks following antigen exposure (Wang *et al.*, 2000). It is therefore possible that, over the course of 28 days, the mice may have mounted an IgG response to the dosed antibody. The resultant immune complexes, consisting of murine IgG bound to rat IgG, may then have activated murine DCs via FcγRs, resulting in the observed widespread DC maturation, as evaluated by CD86 expression, in all experimental groups, regardless of the specificity of the rat IgG.

OX40L expression by DCs was also assessed at day 28 following bleomycin challenge or saline treatment. OX40L is not expressed by immature or quiescent DCs, but is upregulated following activation (Redmond *et al.*, 2009). The mediators and mechanisms regulating OX40L expression by DCs remain poorly defined. TSLP represents the only known cytokine capable of upregulating DC OX40L expression, though ligation of CD40 receptor on DCs by its cognate ligand, CD40L, expressed by activated T-cells, has been reported to result in increased OX40L expression (Ohshima *et al.*, 1997). The present observation of high OX40L expression in all experimental groups, including saline, again suggests an effect mediated by the administration of rat IgG, over a prolonged dosing schedule, which has resulted in widespread DC activation. However, given that TSLP has recently been identified as a key inducer of OX40L expression by DCs, the reduction in the proportion of OX40L+ DCs in mice receiving anti-TSLP antibody potentially suggests successful target engagement in this model. Despite this, however, no reduction in CD86

expression was observed in mice receiving anti-TSLP antibody. This may be explained by the presence of multiple inducers of CD86 expression during this phase of lung remodelling, which may include other danger signals, including uric acid and eATP, or endogenous proteins rendered immunogenic following lung injury. Further studies are required to conclusively demonstrate that DC activation following bleomycin-induced lung injury is maintained during later time points, and furthermore, to identify possible candidates responsible for such activation.

In summary, although the present studies demonstrate the importance of TSLP in mediating bleomycin-induced DC activation during the inflammatory phase of this model, it is currently not possible to draw similar conclusions regarding the role of TSLP in DC activation during the later fibrotic phase of this model.

4.6.2. TSLP mediates bleomycin-induced polarisation of the immune response towards a T-2 phenotype

There is strong evidence that bleomycin-induced lung injury results in polarisation of the immune response towards a T-2 phenotype and targeting this polarisation attenuates bleomycin-induced lung fibrosis (Belperio *et al.*, 2002). Although numerous studies have demonstrated the importance of T-cells to the pathogenesis of experimentally-induced fibrosis, these findings are not universal (Helene *et al.*, 1999; Piguet *et al.*, 1989). However, a recent study demonstrated that mice deficient in Nrf2, a key transcriptional regulator of cellular anti-oxidant defences, developed worse fibrosis following bleomycin-induced lung injury than their wild-type controls - this effect was associated with an increase in lung Th2 cells (Kikuchi *et al.*). Induction of a Th2 bias in mice overexpressing GATA-3, the master Th2 transcription factor, was also associated with a worsening of experimentally-induced fibrosis (Kimura *et al.*, 2006). Nonetheless, polarisation of T-cell subsets into Th1 or Th2 phenotypes has not been extensively evaluated in these models. Although human studies have hinted at a polarisation of T-cell subsets in IPF towards a T-2 phenotype (Pignatti *et al.*, 2006), a causal relationship between Th2 cells and lung fibrogenesis remains to be established.

To the best of my knowledge, the findings from the current studies demonstrate for the first time clear polarisation of the T-helper population towards a T-2 phenotype following bleomycin-induced injury. This response is observed during both the inflammatory and fibrotic phases of this model. The observed polarisation is local and limited to the lung, as no such differences were observed in splenic T-cells of injured mice and importantly was TSLP-dependent. As described in **Section 4.6.1.**,

the majority of DCs expressed OX40L in both saline-treated and bleomycin-challenged mouse groups. However, only bleomycin-challenged mice demonstrated an accumulation of Th2 cells in the lung. Despite the importance of OX40L-expressing DCs in inducing Th2 cell differentiation, these findings may be reconciled considering the antigen-specific nature of this interaction. As discussed in **Section 4.3.2.**, it is possible that bleomycin-induced lung injury induces accessibility of previously sequestered cellular antigen. Only under these circumstances would TSLP-DCs, expressing OX40L, be in a position to induce a programme of Th2 differentiation. In contrast, the immunogenicity of mice receiving saline treatment is likely to be unchanged and thus OX40L-expressing DCs in this scenario would be unable to induce T-2 polarisation.

These data further, therefore, highlight for the first time the importance of TSLP in the development of a T-2 immune response in the context of non-allergen driven lung disease. As has been discussed previously, TSLP may promote expansion and differentiation of Th2 cells indirectly, via DCs, or via direct actions on naïve or memory T-cells. At the current time, it is not possible to determine which pathway predominates in this model. The observation that inhibition of TSLP activity *in vivo* is associated with a concomitant reduction in the proportion of activated DCs at day 10 post bleomycin-induced injury suggests that TSLP-DCs are at least in part involved in directing the local T-helper response. This issue will be further explored in **Section 4.9.1.**

Although bleomycin challenge induced a TSLP-dependent increase in the proportion of Th2 CD4⁺ cells, no changes were observed in the total TCRβ⁺ T-cell, nor the total CD4 or CD8 cell populations. This is in contrast to previous studies demonstrating an increase in lung T-cells following bleomycin challenge (Zhu *et al.*, 1996). This discrepancy may be explained by the fact that in the present study, T-cells were assessed in whole lung homogenates, in contrast to BALF specimens used in previous studies. The use of BALF specimens circumvents the problem of contamination of samples by circulating T-cells derived from the vasculature at the time of harvest, but do not permit analysis of effector T-cells organised within aggregates in areas of fibrotic lung parenchyma, as found in fibrotic lung. Moreover, the cellular yield is much lower, which limits the extent of FACS analysis. In the current studies, each mouse lung was used for the preparation of cells for positive and negative controls, as well as for both DC and T-cell analysis. Moreover, cell loss following stimulation of cells with PMA and ionomycin was a major issue during

the course of these experiments. Lung perfusion via the pulmonary vasculature is often used to enable the generation of single cell suspensions from lung homogenates which are not contaminated by vascular-derived cells. However, the pattern of lung damage following bleomycin-induced injury makes it highly unlikely that any lung perfusion would be uniform and consistent. In view of the requirement of sufficient cell numbers to analyse Th1 and Th2 cell populations, as well as DCs, single cell preparations were generated from non-perfused lung homogenates as described in **Materials and Methods**.

In summary, the present studies demonstrate for the first time that bleomycin-induced lung injury is associated with a polarisation of the T-helper population during the inflammatory phase, which is maintained during the fibrotic phase of this model. Moreover, they provide evidence for a role for TSLP as a master switch for the development of T-2 immune responses beyond allergic inflammation. In light of the strong evidence suggesting that the development of such responses is associated with the development of fibrosis, the effect of redressing this polarisation of T-helper cells on the development of lung fibrosis following bleomycin-induced lung injury will now be discussed.

4.7. The role of TSLP in the pathogenesis of bleomycin-induced lung fibrosis

The present studies have identified lung fibroblasts as a potential source of TSLP and cellular target in both IPF and bleomycin-induced lung fibrosis. As discussed in **Section 4.3.**, there are multiple pathways via which TSLP may play a role in the pathogenesis of lung fibrosis. In particular, the current findings demonstrate that neutralisation of TSLP activity abrogates bleomycin-induced T-2 polarisation of T-helper cells, thereby inhibiting the development of a key cellular source of T-2 cytokines. In light of the strong evidence suggesting that the development of lung fibrosis is dependent, to a greater or lesser degree, on the expression of such pro-fibrotic T-2 cytokines (Belperio *et al.*, 2002), I hypothesised that this abrogation would be associated with an attenuation in bleomycin-induced lung fibrosis in mice. The results described in **Section 3.4.7.** will now be discussed in more detail.

4.7.1. The effect of 28F12 administration on bleomycin-induced lung fibrosis

Contrary to our expectations, neutralisation of TSLP activity *in vivo*, despite reducing the presence of Th2 cells in the lung, was not associated with an

attenuation in bleomycin-induced lung fibrosis, as assessed by total lung collagen at days 14 or 28 (**Section 3.4.7.**). These observations suggest that TSLP does not contribute to lung collagen accumulation in this model. However, it is important to interpret these findings with a degree of caution as these data may be confounded by the overwhelming activation of DCs seen in the context of repeated antibody dosing over 28 days. Aside from inducing T-cell differentiation and proliferation, activated DCs may potentially contribute to the development of fibrosis by alternative mechanisms, including the generation of chemokine gradients (**Section 4.3.2.**). However, if these data do represent a true negative result, they are unexpected for a number of reasons. T-2 cytokine activity has previously been demonstrated to be required for the development of bleomycin-induced fibrosis *in vivo* (Belperio *et al.*, 2002), and the downstream effects of TSLP have been shown to be mediated by these cytokines (Zhou *et al.*, 2008). Moreover, both IL-4 and IL-13 have been shown to exert strong pro-fibrotic effects *in vitro* (Saito *et al.*, 2003). Reducing the predominance of Th2 cells in the lung by neutralising TSLP activity *in vivo* may therefore have been expected to reduce the local elaboration of such pro-fibrotic cytokines.

The lack of protection afforded by the administration of 28F12 from bleomycin-induced fibrosis, despite apparent successful engagement with TSLP, may have a number of explanations. First, it is possible that in this particular model, the development of lung fibrosis is independent of the phenotype of the immune response. Previous studies targeting T-2 cytokine activity have been performed in mice administered bleomycin via the intra-tracheal (i.t.) route (Belperio *et al.*, 2002; Jakubzick *et al.*, 2003), rather than via the oro-pharyngeal (o.p.) route employed in the current studies. Although bleomycin is administered via the airways in both models, it is clear that the pattern of fibrosis is different. Following a surgical procedure, i.t. administration results in a more bronchocentric pattern of lung fibrosis, in contrast to the more peripheral and sub-pleural pattern observed following o.p. challenge, which does not involve surgical trauma to the airway. The o.p. model was chosen for the current studies, as the pattern of fibrosis was felt to more faithfully mirror that found in human IPF. However, the spatial and temporal heterogeneity of IPF is critical to its diagnosis, and different geographical patterns of fibrosis in humans often reflect different underlying pathogenetic mechanisms, including the relative predominance of immune responses. This concept may be translated back to murine models of disease, and reflects an inherent issue of generating meaningful comparisons between different studies. For instance,

depending on the route of administration, bleomycin-induced lung fibrosis has been reported to either completely resolve by 6 weeks or be maintained at 6 months following injury (Scotton *et al.*, 2010). These differences are profound and are likely to reflect the predominance of certain molecular pathways in different versions of the “same model”.

In addition, the difficulty in comparing data from different studies is further compounded by variations in dose, mouse strain and measurements of fibrosis outcomes. Previous studies demonstrating an attenuation in bleomycin-induced fibrosis following interference in T-2 cytokine signalling have employed CBA/J mice (Belperio *et al.*, 2002; Jakubzick *et al.*, 2003), in contrast to the present studies which employed C57Bl/6 mice challenged with bleomycin. These mice were chosen for the present studies in light of their well-characterised response to bleomycin in the host laboratory. However, significant differences in the immune responses in these two strains have been reported in models where the nature of the immune response is arguably more directly related to the pathogenesis of the target condition, for instance in infection models. For example, mice of a C57Bl background generate significantly lower quantities of IL-4 than CBA/J mice following *Leishmania* infection (Rosas *et al.*, 2005). In addition, C57Bl mice are relatively resistant to infection with *Mycobacterial tuberculosis*, a characteristic related in part to differential expression of IL-10, an important immunomodulatory cytokine (Turner *et al.*, 2001). Further differences between mouse strains which may have impacted on the present studies, include variability in T-cell recruitment and maintenance in the lung (Chackerian *et al.*, 2003), and strain-dependent differences in DCs have also been reported to influence CD4+ T-cell polarisation (Filippi *et al.*, 2003).

In the current studies, lung collagen was assessed by measuring hydroxyproline content in lung powder by reverse-phase HPLC. Previous studies evaluating the importance of T-2 immune responses have utilised colometric assays to measure collagen (Belperio *et al.*, 2002). A major issue with such assays is that they measure only soluble collagen, in contrast to HPLC which takes into account insoluble collagen, arguably a more biologically relevant readout in the context of fibrotic lung disease. Ongoing studies within the host laboratory have revealed that these two read-outs do not necessarily correlate over the time course of the bleomycin model (Scotton *et al.*, manuscript in preparation). Thus, it is apparent that there a number of reasons, both operator-dependent and -independent, which may potentially explain the differences between the current studies and previous reports

highlighting the importance of a T-2 immune response in the pathogenesis of bleomycin-induced fibrosis.

Alternative cellular sources of T-2 cytokines may also explain the lack of protection afforded by reducing the predominance of Th2 cells in this model. IL-13 was originally described as a T-cell derived cytokine (Minty *et al.*, 1993), though it is now well recognised that other cells are capable of generating IL-13, including mast cells (Toru *et al.*, 1998), basophils (Li *et al.*, 1996), eosinophils (Schmid-Grendelmeier *et al.*, 2002), macrophages (Hancock *et al.*, 1998) and epithelial cells (Allahverdian *et al.*, 2008). More recently, a novel innate leukocyte effector cell, termed a nuocyte (Neill *et al.*, 2011), has been described as an important cellular source of this cytokine. It is possible that in the pathogenesis of lung fibrosis, Th2 cells are not the predominant source of T-2 cytokines. The predominant cellular source of T-2 cytokines in this model may therefore include cells whose expression of these cytokines is not ultimately regulated by TSLP. Of these, no pathogenetic role for nuocytes has yet been suggested in the development of lung fibrosis. The importance ascribed to the macrophage in lung fibrogenesis has waxed and waned over recent years, though there is now renewed interest in this effector cell in IPF (Baran *et al.*, 2007; Prasse *et al.*, 2006). In addition, it has been proposed that the macrophage represents a major cellular source of TGF- β in experimentally-induced fibrosis (Khalil *et al.*, 1989). In particular, alternatively activated macrophages, a phenotype preferentially induced by T-2 cytokines (Stein *et al.*, 1992), are associated with the development of fibrosis in both human disease and animal models (Varin *et al.*, 2009; Wynn, 2004), including lung fibrosis (Murray *et al.*). With relevance to the present studies, there are reports that macrophages may represent an important source of IL-13 in fibrosis (Hancock *et al.*, 1998), though the regulation of macrophage IL-13 production remains unclear. Epithelial cells have also been reported to be capable of expressing IL-13 in response to mechanical injury (Allahverdian *et al.*, 2008), which is of particular interest given the perceived importance of epithelial injury in initiating both experimentally-induced fibrosis and IPF. Eosinophils have also been demonstrated to be a major source of T-2 cytokines in the bleomycin model (Huaux *et al.*, 2003b), and may therefore represent another cell type whose expression of pro-fibrotic T-2 cytokines might be unaffected by neutralising TSLP activity *in vivo*. To the best of my knowledge, the current studies are the first to demonstrate clear polarisation of the T-helper cell populations in the lung following bleomycin-induced lung injury. However, the current studies did not evaluate the expression of T-2 cytokines at the protein level

in this model. Thus, while neutralisation of TSLP activity *in vivo* may reduce the prevalence of effector Th2 cells in the lung following bleomycin injury, one would not expect this intervention to inhibit the continued local production of T-2 cytokines by macrophages, epithelial cells and eosinophils, which may continue to promote a pro-fibrotic microenvironment. The current findings are compatible with findings from studies evaluating the role of TSLP in certain helminth-induced T-2 immune responses. In these studies, despite a significant reduction in T-2 cytokine expression by Th2 cells in TSLPR^{-/-} mice undergoing secondary granuloma formation in the lung, the size of granuloma was unaffected compared to wild-type controls (Ramalingam *et al.*, 2009).

In summary, the present studies suggest that the development of bleomycin-induced fibrosis is independent of both TSLP activity and the phenotype of T-helper cells in the lung. However, it is not possible at the current time to draw any conclusions regarding the dependence of fibrogenesis in this model to a T-2 phenotype in general. Further studies evaluating protein expression of T-2 cytokines would be needed to address this issue and will be discussed in **Section 4.9.1**.

4.8. Conclusion and implications

This thesis examined the hypothesis that TSLP is upregulated in the fibrotic lung and contributes to fibrogenesis by promoting the development of a T-2 immune response following epithelial injury. The work presented herein reports for the first time strong TSLP and TSLPR immunoreactivity in bleomycin-induced lung fibrosis and IPF. These findings were extended *in vitro* to demonstrate that the human lung fibroblast may represent an important cellular source of TSLP in response to signals relayed from an injured epithelium. Moreover, the present studies demonstrate the presence of a hitherto unreported functional TSLP-TSLPR signalling axis in lung fibroblasts, further highlighting the potential of this cell to act in an immunoregulatory manner. The importance of TSLP as a key mediator of immune responses characterised by a predominance of Th2 cells was extended beyond non-allergen driven lung disease, and TSLP was identified as a critical mediator of DC activation following bleomycin-induced lung injury. However, these studies revealed that the development of lung fibrosis in this model is independent of a predominance of Th2 cells in the lung, and suggest that, while a T-2 immune response to lung injury may be pro-fibrotic, alternative cellular sources of T-2 cytokines may contribute to the pathogenesis of lung fibrosis.

The pathogenetic role of T-cells in lung fibrosis remains a contentious issue, with different studies in animals yielding contradictory results. Moreover, despite reports of functional differences in T-cell populations from IPF and control patients, a causal relationship between the adaptive immune system and the pathogenesis of fibrosis has yet to be defined in this condition. It is increasingly clear, however, that the heterogeneity of mechanisms through which T-cells may participate in the immune response to epithelial injury, means that universal depletion or suppression of T-cell activity is unlikely to be beneficial to patients. Rather, phenotypic modulation of T-cells in the lung may offer more promise as a therapeutic strategy. Although the current studies failed to demonstrate such an effect in bleomycin-induced lung fibrosis, it is important to acknowledge the difficulties in translating results from animal models to human disease. The bleomycin model is a useful model to delineate the relative importance of potential pro-fibrotic mechanisms but, as has been well documented, is by no means an accurate representation of all the features of IPF (Scotton *et al.*, 2010). Aside from pirfenidone and NAC, optimism that targets derived from attenuation of experimentally-induced fibrosis would translate into a clinical benefit in IPF has not yet been realized. Nonetheless, the current studies suggest that in IPF, T-cells may not represent the predominant

cellular source of pro-fibrotic cytokines. As has been described, both alveolar epithelial cells and macrophages are capable of elaborating IL-13, and the relative importance of these cellular sources in IPF remains undefined. However, modulation of the immune phenotype in this condition remains a potentially successful therapeutic option and trials evaluating the use of an anti-IL-13 antibody in IPF are currently ongoing (QAX576; Novartis, Switzerland).

While the current studies have failed to demonstrate a clear pathogenetic role for TSLP in the development of bleomycin-induced lung fibrosis, they do support the notion that the lung fibroblast represents a potential immunoregulatory cell, capable of modulating the nature of the immune phenotype at sites of injury. In particular, the work presented in this thesis supports the notion that fibroblasts are capable of acting as sentinel cells, responding to tissue injury by influencing immune cell recruitment; the scientific rationale for targeting this aspect of fibroblast biology in inflammatory lung diseases characterised by excessive activity of the adaptive immune system is rapidly gaining strength.

In conclusion, IPF remains a devastating and progressive condition with an appalling prognosis. It is clear that the fibroproliferative response to injury seen in this condition reflects an extremely complex interplay between a number of different cellular and signalling mechanisms, with an unknown degree of redundancy. By demonstrating the independence of bleomycin-induced fibrosis from T-cell polarisation, the work presented in this thesis contributes to this field of knowledge by suggesting that the pro-fibrotic “T-2 immune phenotype” perceived as characteristic of IPF may in fact be mediated by cells other than T-lymphocytes. Indeed, the present findings highlight the need for further human studies to determine the relative importance of T-cells to the development of such phenotypes in this condition.

4.9. Future work

4.9.1. Animal models of lung fibrosis

The findings presented in this thesis have provided evidence that the development of bleomycin-induced lung fibrosis is independent of TSLP-mediated DC activation and polarisation of T-helper cells towards a T-2 phenotype. To reconcile these findings with previous reports highlighting the importance of T-2 cytokines in lung fibrosis, further studies would be required to examine the expression of T-2 cytokines in this model. Quantitative evaluation of IL-13 protein expression is technically challenging in murine lung homogenates and to the best of my knowledge has not been reported in the bleomycin model. While it is possible to measure expression at the mRNA level, the interpretation of such studies are hampered in this model by the potential dilution of signal by mRNA emanating from the massive cellular infiltrate into the lung following bleomycin injury. Nonetheless, such studies may help determine if the development of a T-2 immune phenotype occurs independently of T-cells. Concomitant immunostaining for T-2 cytokines in this model may also help resolve this issue but was not attempted due to time constraints. An alternative approach would be to examine T-2 cytokine expression in mice deficient in T- and B-cells challenged with bleomycin. Such depletion may be achieved by anti-CD3 therapy administration to wild type mice or by using mice devoid of T- and B-cells, such as *rag*^{-/-} mice.

While the present studies have demonstrated the importance of TSLP in promoting the predominance of Th2 cells in the lung following bleomycin-induced lung injury, it is not clear whether this effect is mediated by TSLP-DCs, or via a direct effect on T-cells. To resolve this issue, future studies may examine the effect of conditional depletion of lung DCs (van Rijt *et al.*, 2005) in the bleomycin model. Such experiments may also confirm the independence of bleomycin-induced fibrosis from DC activation, as has been suggested by the present studies.

The findings presented in this thesis have demonstrated that immunoreactivity for TSLP in the lung is greater in bleomycin-challenged mice compared to saline-instilled controls, localising to alveolar epithelial cells as well as fibroblasts. From the present studies, it is not possible to determine which of these cell types represents the predominant cellular source in promoting DC activation and the promotion of Th2-dominant lymphocyte population in the lung following bleomycin-induced injury. This question may be addressed by the generation of mice containing a conditional fibroblast-specific deletion of *Tslp*. C57Bl/6 mice which carry an inducible Cre-

recombinase under the control of a fibroblast-specific regulatory sequence, for instance from the $\text{pro}\alpha 2(\text{I})$ collagen gene, could be crossed with mice that carry a floxed TSLP allele. The use of such mice has not, to the best of my knowledge, been reported in the literature but would represent an exciting opportunity to determine the functionally dominant cellular source of TSLP following bleomycin-induced lung injury.

Finally, to address the relative importance of TSLP in mediating DC activation in this model during the fibrotic phase, further studies may be performed using a small molecular weight antagonist of TSLP. Although such a compound is not currently commercially available, the success of such a molecule (*developed by Actimis Pharmaceuticals Inc, USA*) in attenuating cutaneous allergic inflammation suggests that it may offer an alternative effective mechanism to neutralise TSLP activity *in vivo*. Such experiments would circumvent the problems highlighted with repeated antibody dosing in this model and may indeed offer a more complete neutralisation of TSLP activity in the bleomycin model.

4.9.2. Human disease

While the present studies have demonstrated strong immunoreactivity for TSLP and TSLPR in IPF, future studies would be directed towards assaying expression at the protein level in this condition. Although neutralisation of TSLP activity had no effect in an animal model of lung fibrosis, the observation of increased levels of TSLP in IPF patients would corroborate the present immunohistochemical data and strengthen the notion that this cytokine might contribute to the pathogenesis of human disease. In addition, qRT-PCR could be performed on laser-capture microdissected IPF lung tissue to confirm that fibroblasts represent an important source of TSLP in this disease. An alternative approach to confirm the cellular provenance of TSLP in this condition would be to perform combined *in-situ hybridisation* studies with immunohistochemical analysis of IPF sections.

4.9.3. Cell biology

The mechanisms involved in mediating STAT phosphorylation following ligation of the TSLP receptor complex remain unclear. Although the present studies have demonstrated that TSLP induces phosphorylation of STAT3 in pHLFs, the relative importance of JAKs was not addressed and would form a major part of future studies to delineate the signalling pathways downstream of TSLP in this cell type.

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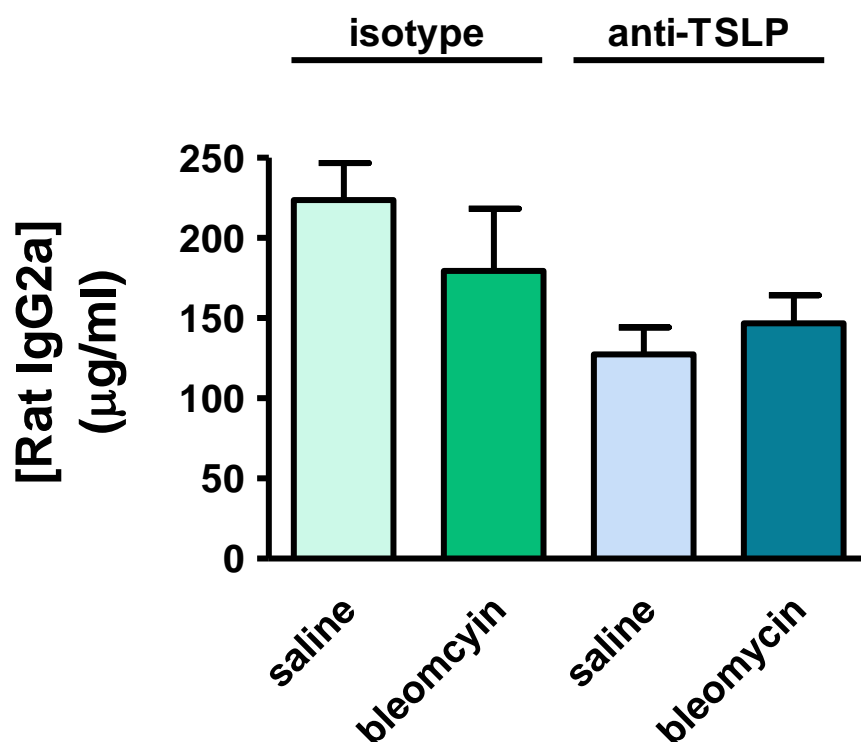
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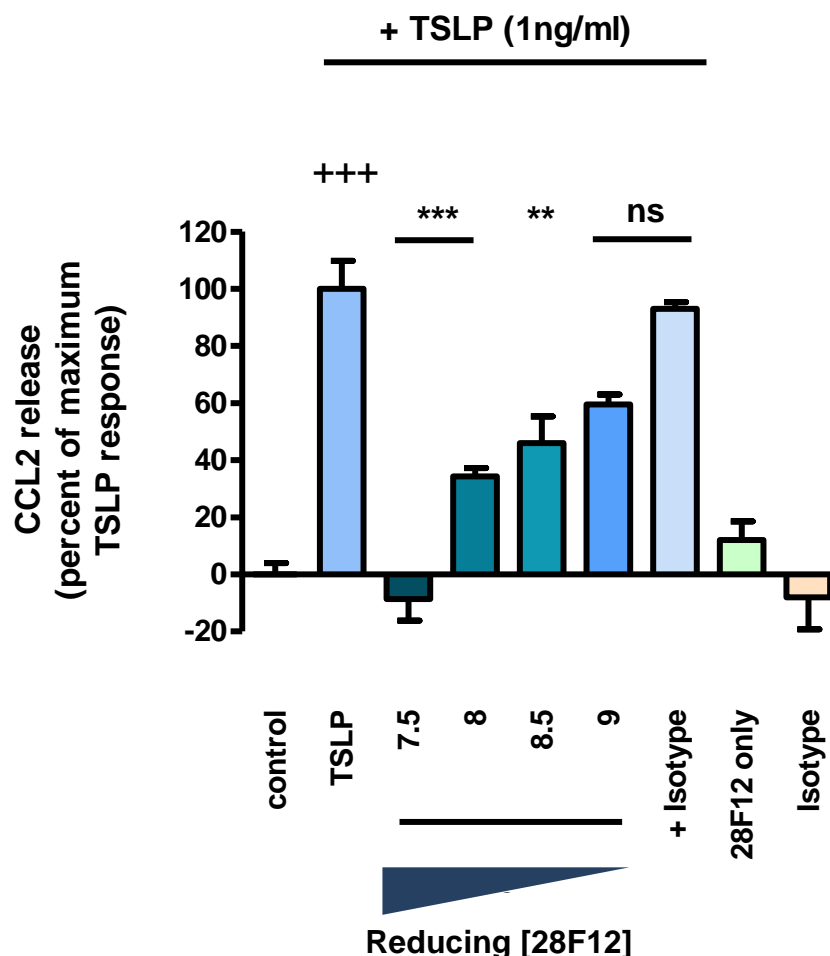
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APPENDICES



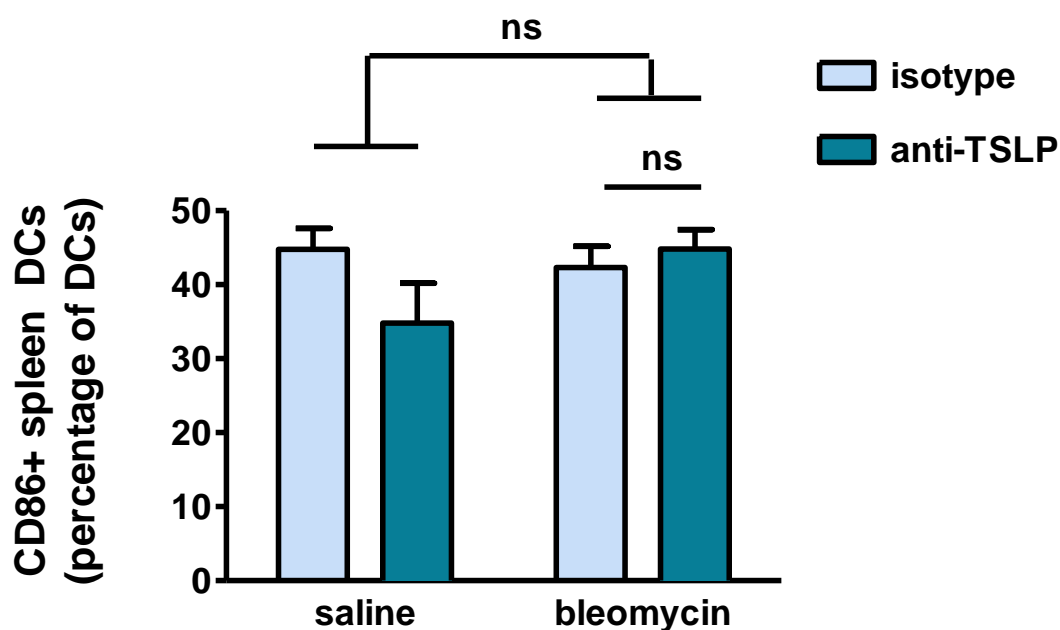
A1 Serum concentrations at day 14 of anti-TSLP and isotype control antibodies following intra-peritoneal injection in mice instilled with saline or bleomycin.

Figure shows the serum concentrations of the monoclonal rat anti-mouse IgG2a TSLP antibody, 28F12, and the isotype control antibody achieved following intra-peritoneal injections (15 mg/kg mouse weight). C57Bl/6 mice were instilled with saline or bleomycin at day 0 as described in **Materials and Methods**. Antibody administration was commenced at day -1, and continued every 4 days until the time of sacrifice at day 14. Serum samples were taken from mice and analysed for antibody levels by ELISA (kindly performed by GSK). The concentration of antibody is expressed as µg/ml, and each bar represents the mean \pm SEM of all mice in each group (n=8, saline/isotype; n=8, saline/anti-TSLP; n=6, bleomycin/isotype; n=8, bleomycin/anti-TSLP).



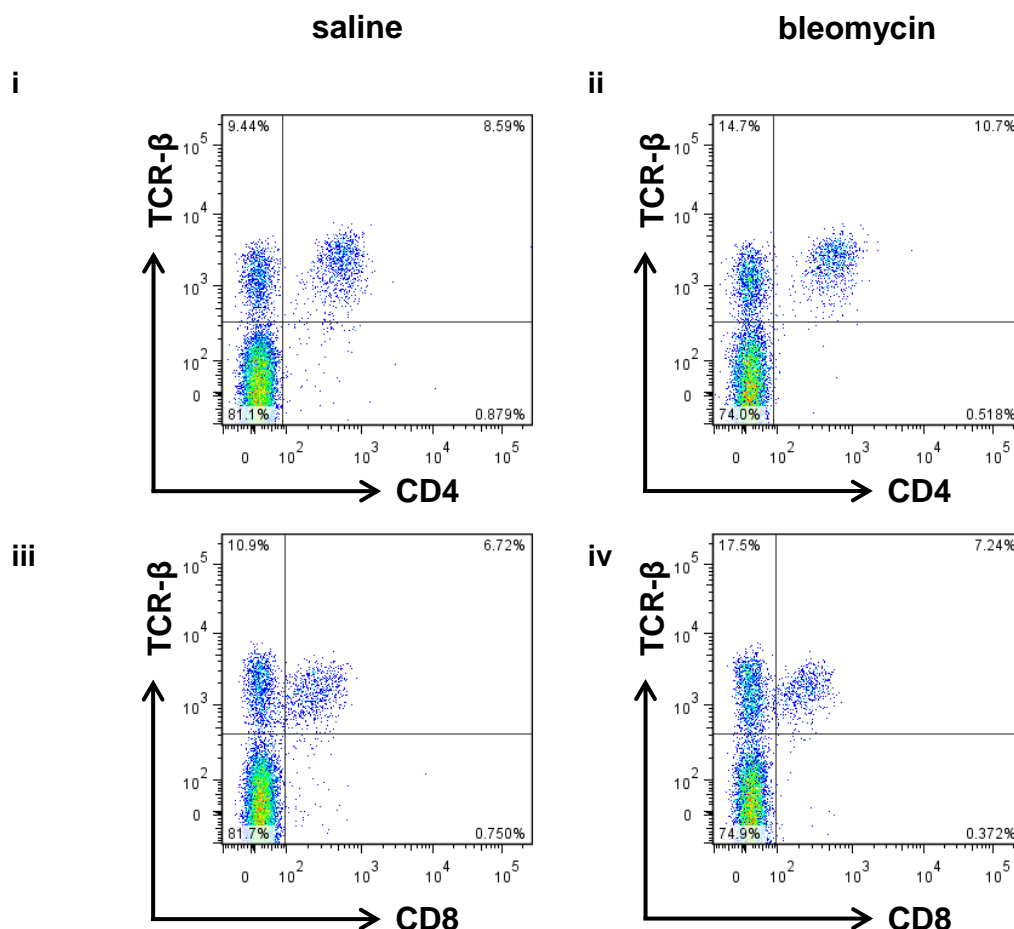
A2 TSLP-induced CCL2 protein release by primary murine lung fibroblasts (pMLFs) is inhibited by the rat monoclonal anti-mTSLP antibody, 28F12.

Figure shows the effect of an anti-mTSLP antibody, 28F12, on TSLP-induced CCL2 protein release by pMLFs. Data are presented as a percentage of the maximal response obtained with mTSLP only. pMLFs were exposed for 6 hours to control medium; isotype control antibody only (30 ng/ml); 28F12 only (30 ng/ml); mTSLP (1 ng/ml) pre-incubated with isotype control antibody (30 ng/ml) or varying concentrations of 28F12 (for 2 hours). Conditioned media were analysed for CCL2 protein release by ELISA. The first bar represents the response to control medium alone. The second bar represents the response to mTSLP only. The final bars represent the response to isotype control and 28F12 antibodies respectively (30 ng/ml), and show that these antibodies had no effect on basal CCL2 release. Negative log of the concentrations of 28F12 (g/l) are presented. Data represent the mean \pm SEM, from triplicates. +++ $p < 0.001$, comparison with control; ns-non significant medium, ** $p < 0.01$, *** $p < 0.001$, comparison with mTSLP alone.



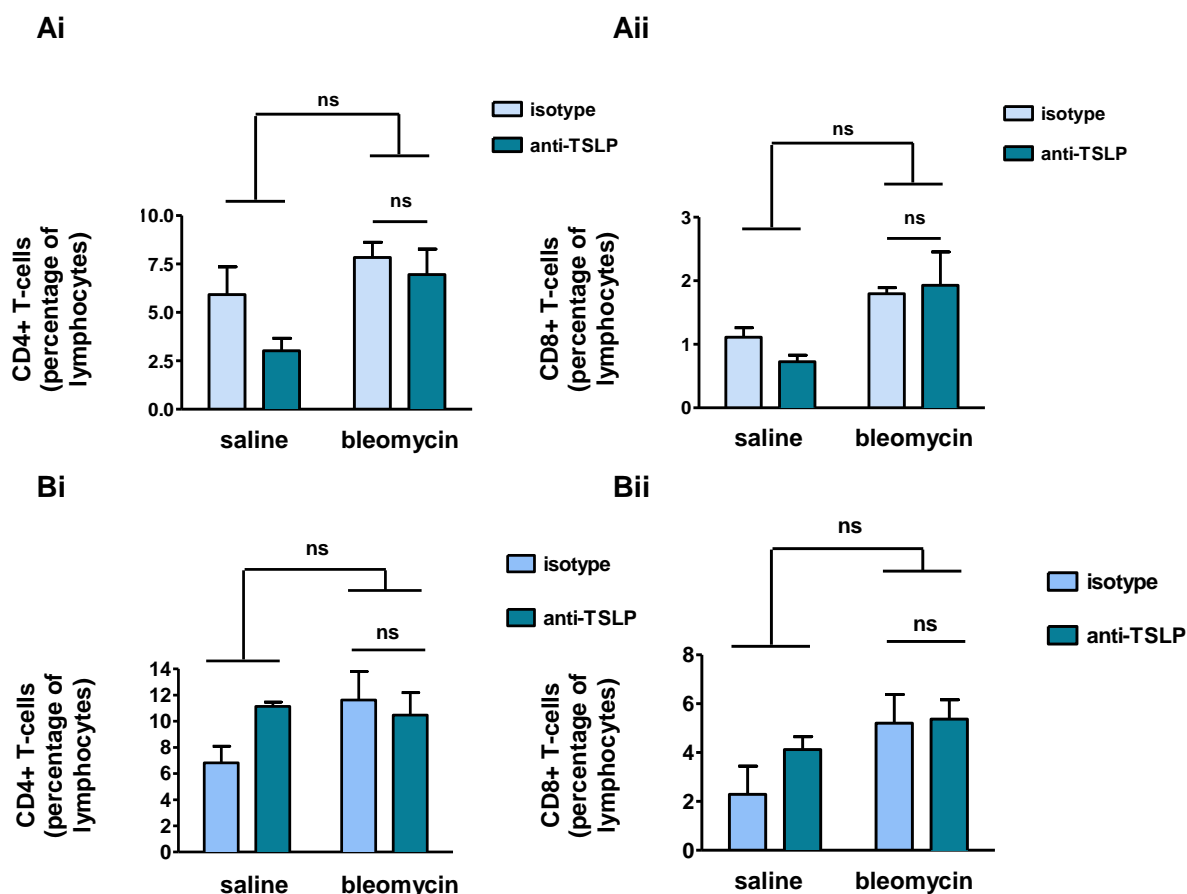
A3 CD86 expression by splenic DCs is similar for saline-treated and bleomycin-challenged mice at day 10.

Figure shows the effect of anti-TSLP administration on CD86 expression by splenic DCs following saline or bleomycin instillation. C57Bl/6 mice were instilled with saline or bleomycin, and administered isotype control or anti-TSLP antibody i.p. as per **Materials and Methods**. Spleens were harvested for preparation of single cell splenic suspensions as described in **Materials and Methods**. Data are presented as percentage of splenic dendritic cells (identified by an identical gating strategy to that employed for lung DCs) which are CD86+ \pm SEM; n=3, all groups; ns – non significant, two-way ANOVA.



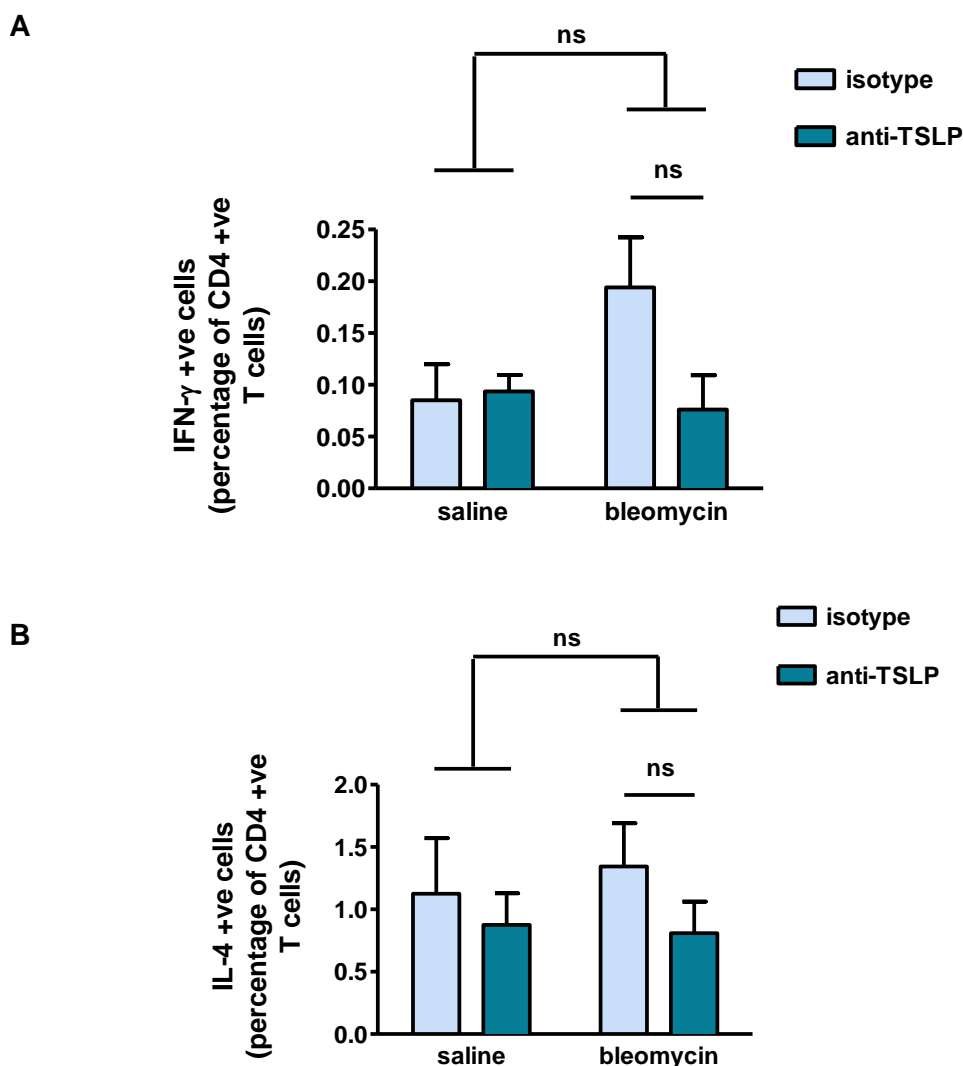
A4 Bleomycin challenge in mice does not result in an increase in the total, CD4+ or CD8+ lung T-cell populations at day 7.

Figure shows representative FACS plots demonstrating lung $\alpha\beta$ T-cell populations 7 days following saline or bleomycin instillation. C57Bl/6 mice were instilled with saline or bleomycin; lungs were harvested at day 7 for preparation of single cell suspensions, as described in **Materials and Methods**, for FACS analysis. Cells were initially gated according to forward and side scatter to identify lymphocytes and exclude debris. CD4 (**Panel A**) and CD8 staining (**Panel B**) was then assessed in TCR- β + cells derived from murine lung instilled with saline (**i and iii**) or bleomycin (**ii and iv**); saline, n=3; bleomycin, n=4.



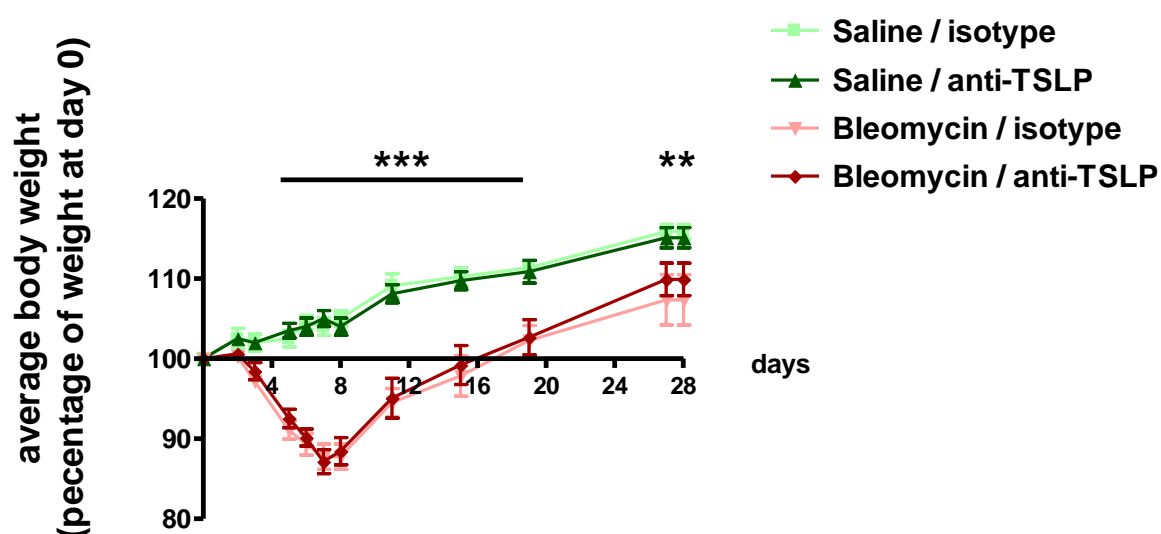
A5 Bleomycin-induced lung injury in mice does not result in an increase in the lung CD4+ or CD8+ T-cell populations at day 10 or day 28.

Figure shows the effect of bleomycin challenge on the CD4+ and CD8+ T-cell populations at day 10 and day 28. C57Bl/6 mice were instilled with saline or bleomycin, and administered isotype control or anti-TSLP antibody i.p.; lungs were harvested at day 10 (**Panel A**) or day 28 (**Panel B**) for preparation of single cell lung suspensions as described in **Materials and Methods**. Cells were initially gated on forward and side scatter to identify lymphocytes, before being gated according to TCR- β and CD4 or CD8 staining. Data are presented as percentage of lymphocytes which are TCR- β + and CD4 +ve (**Ai, Bi**) or CD8 +ve (**Aii, Bii**) \pm SEM.; n=3, saline groups; n=5, bleomycin (day 10); n=3, bleomycin (day 28); ns – non-significant, two-way ANOVA.



A6 Bleomycin-induced lung injury does not result in a change in the Th1 or Th2 phenotype of splenic CD4+ T-cells at day 10 post injury.

Figure shows the effect of bleomycin challenge on IFN- γ -producing/Th1 (**Panel A**) and IL-4-producing/Th2 (**Panel B**) splenic CD4+ T-cells at day 10. C57Bl/6 mice were instilled with saline or bleomycin, and administered isotype control or anti-TSLP antibody i.p.; spleens were harvested for preparation of single cell splenic suspensions as described in **Materials and Methods**. The gating strategy employed to identify TCR- β + /CD4+ cells for analysis of intra-cellular cytokine staining was identical to that used for lung T-cells. Data are presented as the mean percentage of TCR- β + /CD4+ cells which are IFN- γ + (**Panel A**) or IL-4+ (**Panel B**) \pm SEM.; n=3, saline groups; n=5, bleomycin; ns – non-significant, two-way ANOVA.



A7 Effect of 28F12 administration on mouse body weights following bleomycin-induced lung injury over 28 days.

Figure shows the effect of 28F12 administration on mouse body weight following bleomycin challenge over 28 days. C57Bl/6 mice were instilled with saline or bleomycin, and administered isotype control or anti-TSLP antibody i.p. as per **Materials and Methods**. Results are expressed as mean change in body weight as a percentage of the starting weight. *** $p < 0.001$, comparison between saline and bleomycin; repeated measure ANOVA.

A8 Publications and awards arising from this thesis:

Review articles:

Novel therapeutic approaches for pulmonary fibrosis. A Datta, CJ Scotton and RC Chambers. British Journal of Pharmacology. (2011). Novel therapeutic approaches for pulmonary fibrosis. Br J Pharmacol, 163, 141-72.

Abstracts:

TSLP plays a key role in the development of T-2 immune responses in a model of fibrotic lung injury. A Datta, A Williams, R Alexander, CJ Scotton and RC Chambers Am J Resp Crit Care Med ATS supp 2012;

TNF- α induces TSLP expression by human lung fibroblasts in an AP-1 / JNK dependent manner. A Datta, CJ Scotton, A Ortiz-Stern, RJ McNulty and RC Chambers. Am J Resp Crit Care Med ATS supp 2009;

Manuscripts in preparation:

Human lung fibroblasts express a functional TSLP-TSLPR signalling axis. A Datta, CJ Scotton, A Ortiz-Stern, RJ McNulty and RC Chambers

TSLP mediates lung dendritic cell activation and polarisation of the immune response towards a T-2 phenotype following non-allergen driven lung injury. A Datta, A Williams, A Ortiz-Stern, R Alexander, CJ Scotton and RC Chambers

Presentations:

Forthcoming abstract and poster discussion: TSLP plays a key role in the development of T-2 immune responses in a model of fibrotic lung injury; *American Thoracic Society conference, 2012;*

Abstract and oral presentation: The role of TSLP in the development of T-2 immune responses following bleomycin-induced lung injury; *UK Respiratory Research Collaborative Meeting, Royal Society of Medicine 2012;*

Abstract and oral presentation: Human lung fibroblasts express a functional TSLP-TSLPR signalling axis; *British Association for Lung Research (BALR) Meeting 2011.*

Academic achievements:

Winner of Clinical Science Oral Communication Prize, UK Respiratory Research Collaborative Meeting, Royal Society of Medicine (2012);

Runner up BALR Young Researcher of the Year (2011);

Wellcome Clinical Research Training Fellowship (2008).

A9 Ethics approvals for human studies

South West Multi-centre Research Ethics Committee

Dr Helen Booth
Department of Thoracic Medicine
The Middlesex Hospital
Mortimer Street
London
WC1N 8AA

The Lescaze Offices
Shinner's Bridge
Dartington
Devon
TQ9 6JE

Tel: 01803 861947
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Email: swmrec@sw-devon-ha.swest.nhs.uk

8 June 2001

Dear Dr Booth

Re: MREC/01/6/39 – The Pathogenesis of Pulmonary Fibrosis.

The Chairman of the South West MREC has considered the amendments submitted in response to the Committee's earlier review of your application on 10 May 2001 as set out in our letter dated 17 May 2001. The documents considered were as follows:

- MREC Application Forms dated 30 March 2001 and 31 May 2001.
- Study Protocol (4 pages dated by SW MREC as 9 April 2001).
- Patient Information Sheet - Non Fibrotic (H Booth 3.2001 Version 1).
- Patient Information Sheet - Non Fibrotic (H Booth 5.2001 Version 2).
- Consent Form – Non Fibrotic (H Booth 3.2001 Version 1).
- Consent Form – Non Fibrotic (H Booth 5.2001 Version 2).
- Patient Information Sheet – Fibrotic (H Booth 3.2001 Version 1).
- Patient Information Sheet – Fibrotic (H Booth 5.2001 Version 2).
- Consent Form (H Booth 3.2001 Version 1).
- Consent Form (H Booth 5.2001 Version 2).
- CV – Dr Helen Booth.
- Annexe Cs (Mr Dussek, Mr Magee, Mr Walesby and Mr Kolvekar).

The Chairman, acting under delegated authority, is satisfied that these accord with the decision of the Committee and has agreed that there is no objection on ethical grounds to the proposed study. I am, therefore, happy to give you our approval on the understanding that you will follow the conditions of approval set out below. A full record of the review undertaken by the MREC is contained in the attached MREC Response Form. The project must be started within three years of the date on which MREC approval is given.

Conditions of Approval

- No research subject is to be admitted into the trial until agreement has been obtained from the appropriate local research ethics committees.
- You must follow the protocol agreed and any changes to the protocol will require prior MREC approval.
- If projects are approved before funding is received, the MREC must see, and approve, any major changes made by the funding body. The MREC would expect to see a copy of the final questionnaire before it is used.
- You must promptly inform the MREC and appropriate LRECs of:
 - (i) deviations from or changes to the protocol which are made to eliminate immediate hazards to the research subjects;
 - (ii) any changes that increase the risk to subjects and/or affect significantly the conduct of the research;
 - (iii) all adverse drug reactions that are both serious and unexpected;
 - (iv) new information that may affect adversely the safety of the subjects or the conduct of the trial.
- *You must complete and return the standard progress report form to the MREC one year from the date on this letter and thereafter on an annual basis. This form should also be used to notify the MREC when your research is completed.*

While the MREC has given approval for the study on ethical grounds, it is still necessary for you to obtain management approval from the relevant Clinical Directors and/or Chief Executive of the Trusts (or Health Boards/HAs) in which the work will be done.

Local Submissions

It is your responsibility to ensure that any local researcher seeks the approval of the relevant LREC before starting their research. To do this you should submit the appropriate number of copies of the following to the relevant LRECs:

- this letter
- the MREC Application Form (including copies of any questionnaires)
- the attached MREC response form
- Annex D of the Application Form
- **one** copy of the protocol
- the final approved version of the Patient Information Sheet and Consent Form

It is important to check with the respective LRECs the precise numbers of copies required as this will vary and failure to supply sufficient copies could lead to a delay. In addition, you should submit to LRECs only the revised paperwork reflecting the requirements of the MREC as referenced in the response form.

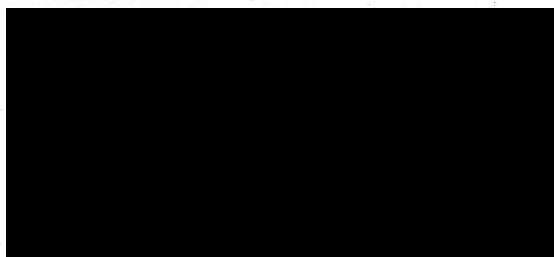
Local Sites

Whilst the MREC would like as much information as possible about local sites at the time you apply for ethical approval it is understood that this is not always possible. You are asked, however, to send details of local sites as soon as a researcher has been recruited. This is essential to enable the MREC to monitor the research it approves.

ICH GCP Compliance

The MRECs are fully compliant with the International Conference on Harmonisation/Good Clinical Practice (ICH GCP) Guidelines for the Conduct of Trials Involving the Participation of Human Subjects as they relate to the responsibilities, composition, function, operations and records of an Independent Ethics Committee/Independent Review Board. To this end it undertakes to adhere as far as is consistent with its Constitution, to the relevant clauses of the ICH Harmonised Tripartite Guideline for Good Clinical Practice, adopted by the Commission of the European Union on 17 January 1997. The Standing Orders and a Statement of Compliance were included on the computer disk containing the guidelines and application form and are available on request or on the Internet at <http://dSPACE.dial.pipex.com/mrec>.

Yours sincerely



Barrie Behenna
Chairman

cc Professor Geoffrey Laurent, Director of Respiratory Research UCL, Rayne Institute, 5 University Street, London WC1N 8AA.

Enclosure:

1. MREC Response Form.



The Joint UCL/UCLH Committees on the Ethics of Human Research (Committee A)

Research & Development Directorate
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London W1P 9LL
POSTAL ADDRESS
Ground Floor, Rosenheim Wing Postroom
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Facsimile: 020 7380 9937

GS/cw/06A278

26th July 2006

Dr Toby M Maher

Wellcome Trust Clinical Research Fellow
University College London
Centre for Respiratory Research
Rayne Institute
5 University Street
London WC1E 6JJ

Dear Dr Maher



Full title of study: Regulation of lung fibroblast and epithelial cell apoptosis by cyclooxygenase-2 and prostaglandin E2 and their role in the pathogenesis of pulmonary fibrosis.

REC reference number: 06/Q0505/48

Thank you for your letter of 18th July 2006, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation [as revised].

Ethical review of research sites

The Committee has designated this study as exempt from site-specific assessment (SSA). There is no requirement for [other] Local Research Ethics Committees to be informed or for site-specific assessment to be carried out at each site.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

<i>Document</i>	<i>Version</i>	<i>Date</i>
Application	5.1	18 July 2006
Investigator CV	Dr. Robin McAnulty	
Investigator CV	Dr. Toby Maher	17 May 2006

Protocol	1	17 May 2006
Covering Letter		17 May 2006
Letter from Sponsor	UCL Biomedicine R&D Unit	15 May 2006
Peer Review	Wellcome Trust referee's reports	19 December 2005
Participant Consent Form: Royal Brompton & Harefield NHS Trust Surgical Consent Form		
Response to Request for Further Information		18 July 2006
Letter from Funder	Wellcome Trust	09 January 2006
Letter confirming indemnity arrangements	AON	01 August 2006

Research governance approval

You should arrange for the R&D department at all relevant NHS care organisations to be notified that the research will be taking place, and provide a copy of the REC application, the protocol and this letter.

All researchers and research collaborators who will be participating in the research must obtain final research governance approval before commencing any research procedures. Where a substantive contract is not held with the care organisation, it may be necessary for an honorary contract to be issued before approval for the research can be given.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

06/Q0505/48

Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely


Dr. Geoff Scott
 Chair

Email: caroline.williams@uclh.nhs.uk

Enclosures: Standard approval conditions

Copy to: University College London Biomedicine Unit

The Joint UCL/UCLH Committees on the Ethics of Human Research
Committee Alpha

Research and Development
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Our Ref: 07QL001

11 January 2007

Dr Toby M Maher
Wellcome Trust Clinical Research Fellow
University College London,
Centre for Respiratory Research
Rayne Institute
5 University Street, London
WC1E 6JJ

Dear Dr Maher

Full title of study: A prospective study of epithelial-mesenchymal interactions in Idiopathic Pulmonary Fibrosis
REC reference number: 06/Q0502/78

Thank you for your letter of 28 November 2006, responding to the Committee's request for further information on the above research [and submitting revised documentation].

The further information was considered at the meeting of Chair's Action's of the REC held on 14 December 2006.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation [as revised].

Ethical review of research sites

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the research site(s) taking part in this study. The favourable opinion does not therefore apply to any site at present. I will write to you again as soon as one Local Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at sites requiring SSA.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

<i>Document</i>	<i>Version</i>	<i>Date</i>
Application	5.1	08 August 2006
Application	5.2	28 November 2006
Investigator CV	Toby Michael Maher	07 August 2006
Protocol	1	07 August 2006
Protocol	2	28 November 2006
Covering Letter		08 August 2006
Summary/Synopsis	2	28 November 2006
Letter from Sponsor		03 August 2006
Participant Information Sheet	2	28 November 2006
Participant Information Sheet	1	07 August 2006
Participant Consent Form	1	07 August 2006
Participant Consent Form	2	28 November 2006
Response to Request for Further Information		28 November 2006
Referees report		19 December 2005
Letter from funder		19 January 2006
Statement of indemnity arrangements		03 August 2006
Supervisor CV	Robin John McNulty	07 August 2006

Research governance approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final research governance approval from the R&D Department for the relevant NHS care organisation.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

06/Q0502/78

Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely



Alda Paulo

Committee Alpha Co-ordinator

Enclosures: Standard approval conditions

Copy to:

- Dr Oke Avwenagha
- R&D Department for NHS care organisation at UCLH